CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

Vol. 9 Internal Secretions of the Pancreas

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CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

VOLUME 9

Internal Secretions of the Pancreas

Editors for the Ciba Foundation

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With 100 Illustrations



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THE CIBA FOUNDATION

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PREFACE

Between January 1950 and July 1955 the Ciba Foundation has organized and held on its premises in London, 36 small, international conferences. In the early days the majority of these were on subjects within the field of endocrinology, partly because the Director was a novice in his task and it was easier to concentrate his efforts within fairly well-defined limits, and partly because research on hormones entered almost every other field of biological activity and so afforded an opportunity for the Foundation's promotion of co-operation, not only internationally, but also, and of at least equal importance, between workers in different disciplines.

The range of the Foundation's conferences has spread much further in recent years, but at least one opportunity is found each year to maintain our interest in endocrinological research. A colloquium on "Hormonal factors in carbohydrate metabolism", held in 1952, had defined many unsolved problems and left an impression of advances being made to some purpose in this field. The Director therefore readily agreed to Prof. Young's suggestion, partly stimulated by Prof. de Duve, for a follow-up colloquium on "The nature and actions of the internal secretions of the pancreas". Both in the arrangements for the meeting and for his chairmanship of the proceedings, the Director is happy to acknowledge his real debt to Prof. Young.

To those to whom this book serves as an introduction to the activities of the Ciba Foundation it should be explained that it is an international centre, which is established as an educational and scientific charity under the laws of England. It owes its inception and support to its founder, CIBA Ltd., of Switzerland, but is administered independently and exclusively by its distinguished British Trustees.

The Foundation provides accommodation for scientific

workers who visit London from abroad, organizes and holds international conferences, conducts (in conjunction with the Institut National d'Hygiène) a post-graduate medical exchange scheme between England and France, arranges informal meetings for discussions, awards an annual lectureship, has initiated a scheme to encourage basic research relevant to the problems of ageing, assists international congresses and scientific societies, is building up a library service in special fields, and generally endeavours to give aid in all matters that may promote international co-operation in scientific research.

Leading research workers from different countries and in different disciplines are invited to attend the symposia or colloquia. The size of the group is, however, very strictly limited in order to obtain a free conversational manner of discussion—although the basic time-table of the programme is strictly observed. The smallness of the groups means the exclusion of many workers active and interested in the subjects discussed, and therefore the proceedings of these conferences are published and made available throughout the world.

It is hoped that the papers and discussions in this book will prove not only informative and stimulating, but will also give to readers a sense of participation in an informal and friendly occasion.

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List of those participating in or attending the Colloquium on "The Nature and Actions of the Internal Secretions of the Pancreas," 21st–23rd June 1955

O. K. Behrens	The Lilly Research Laboratories, Indianapolis, Indiana
C. H. Best	Dept. of Physiology, University of Toronto
J. L. R.-Candela	Inst. of Metabolism and Nutrition, Madrid
C. Cavallero	Inst. of Pathological Anatomy and Histology, University of Pavia
C. F. Cori	Dept. of Biological Chemistry, Washington University School of Medicine, Saint Louis, Missouri
Gerty Cori	*Dept. of Biological Chemistry*, Washington University School of Medicine, Saint Louis, Missouri
L. C. Craig	The Rockefeller Inst. for Medical Research, New York
P. A. Desaulles	Biological Dept., CIBA, Basle
C. de Duve	Dept. of Physiological Chemistry, University of Louvain
H. Ferner	Dept. of Anatomy, University of Hamburg
P. P. Foà	Dept. of Physiology and Pharmacology, The Chicago Medical School, Chicago 12
E. Fredericq	Inst. of Physical Chemistry, University of Liège
M. G. Goldner	Isaac Albert Research Inst. of the Jewish Chronic Disease Hospital, Brooklyn, New York
Dorothy Crowfoot Hodgkin	*Chemical Crystallography* Laboratory, University of Oxford
C. von Holt	Dept. of Physiological Chemistry, University of Hamburg
Margaret Kerly	Dept. of Biochemistry, University College, London
R. D. Lawrence	King's College Hospital, London
J. Lens	Organon Laboratories, Oss, Holland
L. Mosca	Inst. of Pathological Anatomy, University of Milan
C. R. Park	Dept. of Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee

R. R. PORTER	National Inst. for Medical Research, Mill Hill, London
P. J. RANDLE	Dept. of Biochemistry, University of Cambridge
E. J. ROSS	Dept. of Medicine, Peter Bent Brigham Hospital, Boston, Massachusetts
F. SANGER	Dept. of Biochemistry, University of Cambridge
W. SCHULZE	Medical Clinic, University of Leipzig
E. W. SUTHERLAND	Dept. of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio
D. F. WAUGH	Dept. of Biology, Massachusetts Inst. of Technology, Cambridge, Massachusetts
F. G. YOUNG	Dept. of Biochemistry, University of Cambridge

CHAIRMAN'S OPENING REMARKS

This meeting is a mixture of physicists, chemists and biologists which might at first sight seem to be a somewhat immiscible one. I am sure that we shall quickly prove the contrary and that all of us will find interest in the subjects discussed by specialists in other fields. I hope that those who are chemists or physicists will not hesitate to enter into discussions concerning biological matters, and conversely. All may have ideas that can usefully be discussed on an occasion such as this.

I hope we are going to discuss without inhibitions, and perhaps in some detail, the control of the secretion of some of the hormones with which we are concerned. This is a subject that has developed considerably since the last Ciba Foundation Colloquium on today's topic, and some interesting measures of disagreement in this field are foreshadowed by the abstracts before us. Though we may not eliminate these differences of opinion as a result of this meeting, I believe that we shall certainly find a great deal of fruitful discussion on matters such as these and indeed many others.

MORPHOLOGICAL STUDIES OF THE DESTRUCTION OF α-CELLS BY CHEMICAL MEANS

HELMUT FERNER
Department of Anatomy, University of Hamburg

THERE is no doubt that only the β-cells of the islets of Langerhans produce insulin. However, as you know, we have good reason to consider the α-cells as the source of glucagon. Both cell types of islets are characterized by the difference in staining ability of their granules. In adult mammals and in adult man the α-cells amount to approximately 20 per cent and the β-cells to 80 per cent of the islet epithelial cells. In newborn infants the islet organ consists of approximately equal numbers of α- and β-cells. The normal α : β-relationship is dependent upon the age of the individual. One obtains this relationship rather precisely in counting the α- and β-cells in 30–50 islets and taking an average. The α : β-relationship is a significant index. A sharp shift of the α : β-relationship to one side or the other is linked with blood sugar changes. Predominance of the β-cells, for example, after destruction of the α-cells or in β-cell adenoma, results in hypoglycaemic conditions. In all types of permanent diabetes mellitus we find a predominance of the α-cells. This may be a relative or an absolute predominance of the α-cells, as, for example, after the destruction of the β-cells by alloxan (Ferner, 1952).

In rats and rabbits, which are the subject of our studies, we found the α-cells always in the peripheral zone of the islets. In rats they form an incomplete covering around the β-cells (Fig. 1). In rabbits the α-cells are found as individual cells or in small groups on the periphery of the islets.

An insight into the metabolic significance of the α-cells may be gained if the α-cells can be selectively destroyed and eliminated. Such experiments have been carried out by a

number of investigators with different chemicals (Campenhout and Cornelis, 1951; Campenhout *et al.*, 1954; Davis, 1952; Goldner, Volk and Lazarus, 1952; Runge, 1954; v. Holt *et al.*, 1955).

Extreme care must be exercised during microscopic examination, because often as a result of handling of tissues an unspecific shrinkage is found on the periphery of the islets, where the α-cells are located. These artifacts might mislead one into believing that the α-cells have been destroyed. Moreover, the entire pancreas must be examined. If part of the α-cells are injured and the others remain intact, a functional impairment cannot be expected, because the islet system has a great functional reserve. On the other hand, if one considers the morphological picture of the actual destruction of the α-cells it is not as impressive as the destruction of the β-cells by alloxan, because the α-cells comprise only one fifth of the islets.

We have microscopically examined the pancreas of many rats and rabbits which have been treated with synthalin A or with *p*-aminobenzenesulphonamido*iso*propylthiodiazole (IPTD). The dose of synthalin administered was 15 mg./kg. intraperitoneally to rats and 6 mg./kg. intramuscularly to rabbits. IPTD was given in doses of 1–2 g./kg. intraperitoneally to rats and orally to rabbits. For the differentiation of the α- and β-cells Gomori's chromhaematoxylin-phloxin method was used.

Both α-cytotoxic drugs act rapidly on the α-cells. In from 1–3 hours after a single dose we observed the first changes in the α-cells, which as a rule after 24 hours have reached a maximum. The first indication of injury is the clumping of the fine α-granules. The α-cells are filled with large, dirty red-stained clumps, between which the pink cytoplasm is visible. After approximately 3–6 hours only a few clumps are discernible. The cytoplasm loses its affinity for phloxin and appears transparent and very clear (Fig. 2). The α-cells now resemble vacuoles with nuclei whose cell bodies are swollen and enlarged (Fig. 3). This is not a question of true

degranulation. However, it is a progressive dissolution of the cell contents with the enlargement of the cell bodies by absorption, apparently of water.

The nuclei of the α-cells remain unchanged for a longer time. Then in the hydropic stage many nuclei are deformed, their outlines are angular and the chromatin structure is coarser and indistinct. Some of the nuclei are pycnotic.

In rats the hydropic state of the α-cells persists for many days after a single dose. It is not evident that the α-cells undergo necrosis and that they disappear in great numbers. However, no signs of a regeneration of α-cells have been observed.

In rabbits it is quite different. If they survive, the majority of treated animals demonstrate a resorption of the injured α-cells. In a few rabbits the α-cells actually disappeared completely. If one examines, microscopically, the pancreas of an animal 1–2 days after treatment, one finds on the periphery of the islets a cleft, where the α-cells were located previously (Fig. 4). The cytologically intact β-cell complex is now separated from the exocrine parenchyma. In the clefts are visible a few red-stained fragments and débris, which are the remnants of the α-cells (Fig. 5).

It is surprising that sometimes 6 hours after the single dose of synthalin or IPTD the α-cells of the rabbit pancreas can actually be made to disappear, and it is interesting to note that this phenomenon occurs only in some of the treated animals in spite of equal dosage and administration.

Later the gap between the exocrine parenchyma and the β-cell complex is closed and the islets present a normal picture in unspecifically stained preparations.

Especially after large doses of synthalin or IPTD the destruction of the α-cells can be demonstrated in a somewhat different manner, which is reminiscent of the destruction of the β-cells by alloxan. The α-cell groups break up into individual cells, which are separated from one another by gaps. They become small, shrunken and hyperchromatic. They stain intensively red or reddish violet. Even these injured

FIG. 1 One half of an islet of Langerhans from a normal adult rat The α-cells on the periphery, indicated by arrows, contain fine granules Gomori's stain.

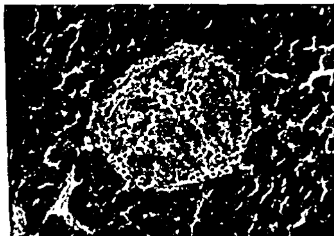


FIG 2 Islet of a rat, 28 hours after a single dose of IPTD; dropic α-cells on the periphery. Low magnification

degranulation. However, it is a progressive dissolution of the cell contents with the enlargement of the cell bodies by absorption, apparently of water.

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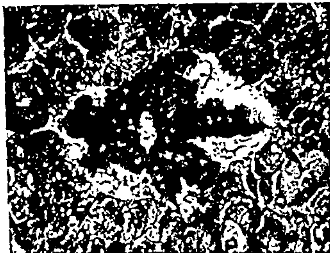


Fig. 5 [illegible]
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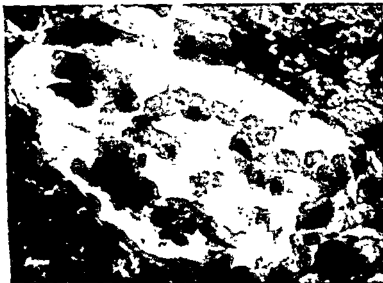


Fig 6 Rabbit treated with IPTD *and* alloxan A destroyed islet containing the remnants of injured β-cells On the periphery are spindle-shaped

Fig. 3 Marked hydropic degeneration and swelling of the α-cells of a rat, 24 hours after IPTD High magnification

Fig. 4 An islet from a rabbit, 6 hours after 2 g /kg. IPTD Destruction and elimination of the α-cells. Clefts with α-cell fragments and débris; β-cells are intact.



Fig. 31 [illegible] mitosis [illegible] and [illegible] administration of 31 mg [illegible]
[illegible]

FIG. 7 Pancreas of a rat treated with alloxan *and* synthalin Loose connective tissue as an islet scar.

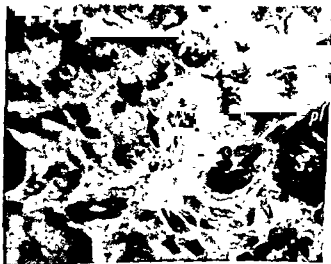


FIG. 8. Pancreas of a rat after combined use of alloxan *and*

α-cells disappear and in their place one finds a cleft with cell fragments.

Concerning the β-cells one cannot demonstrate any lesions after administration of synthalin or IPTD. If the animal does not receive glucose, the β-cells are as a rule small, dense and uniformly granulated. As a consequence of the administration of glucose we observed a degranulation of the β-cells. Glucose was given to offset hypoglycaemic shock, which occurs after administration of synthalin or IPTD.

The combined use of alloxan on the one hand *and* synthalin or IPTD on the other, brings about the destruction of both islet cell systems with the result that the islets of Langerhans completely disappear (Fig. 6). Such a nesectomy will be most completely accomplished by chemical substances, if one commences with a diabetogenic dosage of alloxan and then administers synthalin or IPTD from 5 to 10 days later. When we speak of a complete nesectomy, we mean that in the entire pancreas neither intact islets nor intact islet cells can be observed.

For a short period prior to the complete elimination of the islets one finds scars (Fig. 7). These scars consist of loose connective tissue with a rich capillary network. They exist for a short period of time and then they too disappear completely.

Furthermore, we suppose that the metabolic difference between a total pancreatectomy and a chemical nesectomy, apart from the exocrine pancreas, is related to the existence of a gastro-intestinal α-cell system.

In rats plasmodial α-cell complexes develop after alloxan treatment in the region of the islets. In the duct system, as we know, one observes the outgrowth of α-cell complexes. It is surprising that these newly developed α-cells of alloxan diabetic rats were not injured by α-cytotoxic substances (Fig. 8). Therefore we investigated the α-cells in young rats.

voung rats we have found another effect of synthalin on ...ls (Ferner and Runge, 1955). We gave one subcutaneous ...on of synthalin (10 mg. ... ats ... ays of

life. It was not possible to produce any type of injury to the α-cells in young rats. However, the mitotic frequency of the α-cells in synthalin-treated rats was sharply reduced in comparison with normal animals of the same age. At the fifth day of life, for example, the mitotic rate in treated rats was only 25 per cent of the mitotic rate of normal animals (Fig. 9a). In normal rats the mitotic rate of the α-cells steadily increases from the second to the fifth day of life. In synthalin-treated rats of the same age the curve remains at a low level. The mitotic frequency of the β-cells and of the exocrine cells is the same in treated and in untreated animals (Fig. 9b). A further result was that the existing mitosis of the α-cells in synthalin-treated young rats was, without exception, pathological. The chromosomes of the prophase and the metaphase were clumped and swollen. The entire mitotic figure was pycnotic (Fig. 10).

We conclude that synthalin acts as a selective mitotic poison on the α-cells in young rats during the first days of life without causing noticeable injuries to the α-cells which are not dividing.

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DISCUSSION

Young: Prof. Ferner, you said that in all types of diabetes mellitus there is a predominance of α-cells in the islets. Does that mean that the α : β-cell ratio changes from 1 : 4 to something greater than 1 : 1 in all cases?

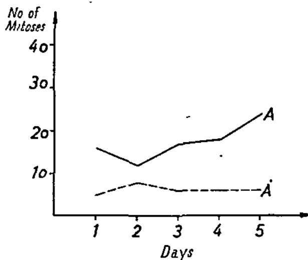


FIG. 9a. A curve showing the mitotic rate of the α-cells in 100 islets of rats from the second to the fifth day of life. A = controls, A˙ = rats after synthalin.

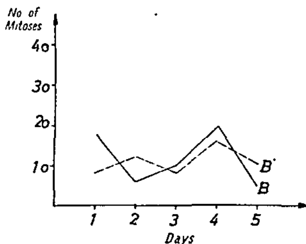


FIG. 9b A curve showing the mitotic rate of the β-cells in 100 islets of rats from the second to the fifth day of life. B = controls, B˙ = rats after synthalin

to the fact that cobalt chloride can produce in the pancreatic islets of the rabbit and the dog histological changes which are almost identical with those shown by Prof. Ferner and produced with IPTD and with synthalin. Shortly after Van Campenhout and Cornelis had demonstrated

most important difference, however, becomes evident if we look at the functional changes in blood sugar homeostasis which accompany the administration of cobalt chloride. There occurs immediately a transitory hyperglycaemia. This was interpreted initially, and as we now know erroneously, as evidence of release of a hyperglycaemic factor from the

glycaemia. These findings led us to conclude that the α-cells do not play a significant rôle in blood sugar homeostasis and that glucagon, if it is produced by α-cells, has little significance for the aetiology of diabetes (1954, *Arch. intern. Med.*, 93, 87)

von Holt: We have also tried to destroy the α-cells by using cobalt

Ferner: There is a relative predominance of α-cells in permanent diabetes mellitus. A ratio of $\alpha:\beta = 2:3$, that means a relative predominance of α-cells, is sufficient, it is not necessarily a ratio greater than 1:1.

Young: Do you know if the gastro-intestinal α-cell system to which you referred is also sensitive to your agents?

Ferner: We do not yet know, because it is very difficult to find the single spread α-cell in the gastro-intestinal mucosa and to be sure that this is one α-cell. This is only possible by silver impregnation methods, and we have no results on it.

Foà: In a seminar at the University of Illinois a year or two ago, Dr. A. A. Godlowski showed that intestinal α-cells may be destroyed by cobalt chloride.

Goldner: I would like to raise two questions with regard to Prof. Ferner's observation of an α-cell preponderance in the pancreatic islets of all types of diabetes. Is it not correct that such α-cell predominance may be found occasionally also in the pancreatic islets of patients with diseases other than diabetes, and that many investigators find it only in the minority of cases with diabetes? I have in mind particularly Gomori's

due to differences in staining techniques? Creutzfeldt (1953, *Beitr.*

only about 6 per cent as α-cells. This silver technique is a modification of the Davenport method which Dr. Volk has developed and which ... than the Gros-

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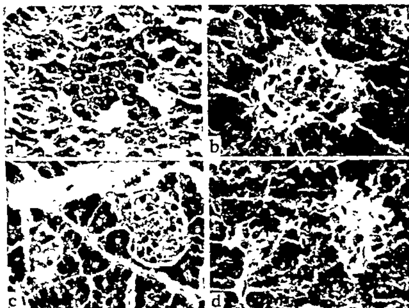


Fig. 1 (Goldner)

(a) Pancreatic islet of a rabbit treated with cobalt chloride. The areas usually occupied by α-cells are empty.

(b) Pancreatic islet of a dog treated with cobalt chloride. Most α-cells have disappeared, a few disintegrating cells are still visible. The arrow points to a capillary filled with blood.

(c) Pancreatic islets of an alloxan diabetic rabbit treated with cobalt chloride. The β-cells show severe nuclear and protoplasmatic damage; there are no α-cells present.

(d) Pancreatic islets of an alloxan diabetic dog treated with cobalt chloride. The areas of both islets are filled by cellular débris.

All slides stained by Gomori's technique.

facing page 10

chloride in doses of 12·5 and 25 mg/kg, and were not ...

differences.

Young: Has it been demonstrated in a depancreatized animal that the α-cytotoxic substance will significantly diminish the intensity of the diabetes?

results. I am familiar with them, and wonder whether the improvement

he histological diagnosis was a tentative one and was based on Gomori ain of the tumour. These cases were characterized by intractable

revealed a protein fraction with mobility characteristics similar to those

Gomori in Chicago in which he states that he was unable to form an opinion from the original slides, but when he received blocks from Dr. Ellison and prepared his own slides, he found that these cells cannot be

Amer. J. clin. Path., 22, 1169). Of course, since nobody thought of this aspect when these cases were reported originally, minor metabolic disturbances may have been overlooked.

argyrophil and stained red with the Gomori method. By using the Hotchkiss method, PAS-positive material has been shown to exist in the tumour cells, a finding which, according to our experience, appears to be characteristic of α-cells. Unexpectedly, neither disturbances of the carbohydrate metabolism nor symptoms of gastric ulcer had been

α-cells?

Ferner: Synthalin injures the liver and the kidney epithelium, but IPTD does not injure the liver or kidney. We have no information about other organs.

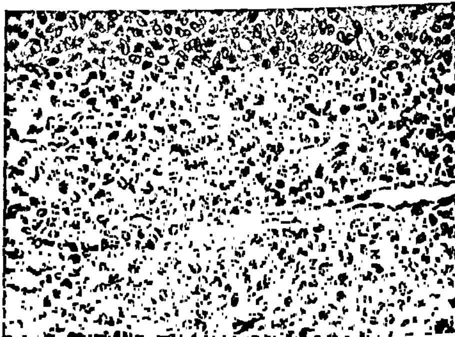


Fig 1 (Cavallero) Low power view of α-cell tumour; irregular arrangement of the cells, with scanty and badly defined cytoplasm.

The histological diagnosis was a tentative one and was based on Gomori stain of the tumour. These cases were characterized by intractable

revealed a protein fraction with mobility characteristics similar to those

tried to find out whether they were associated with disturbances of carbohydrate metabolism. They did not find any evidence (1952, *Amer. J. clin. Path.*, 22, 1169). Of course, since nobody thought of this aspect when these cases were reported originally, minor metabolic disturbances may have been overlooked.

Cavallero: A case of α-cell tumour has been observed by us: the mass, weighing about 500 g, had been slowly developing in the middle portion of the pancreas. Histologically, as shown in Fig 1, it was composed of small-sized, closely packed cells; the cytoplasmatic granula were argyrophil and stained red with the Gomori method. By using the

α-cells?

Ferner: Synthalin injures the liver and the kidney epithelium, but IPTD does not injure the liver or kidney. We have no information about other organs.

von Holt: There is a difference in the behaviour of normal rats wit intact β-cells and those with destroyed β-cells. We have seen that i normal animals the damaged α-cells do not always disappear, but i alloxan diabetic animals the disappearance of these cells is complete Perhaps the altered islet structure after the action of alloxan is the caus of this behaviour.

Foà: Is it possible that one type of cell may change into another?

von Holt: It may be possible, but I am no morphologist and I canno answer you.

Goldner: I have nothing to contribute to the question of transitio from one type of islet cell to another, but I wish to mention that afte alloxan one can observe a reappearance of β-cells in the islets similar t the reappearance of α-cells after α-cytotoxic agents. There is, however, this difference that the α-cells reappear rather soon, after 10 days o 2 weeks, while the β-cells in alloxan diabetic animals may appea only after many months. This has been shown by Lazarow. I do not believe that anyone can say as yet where these new cells come from.

Young: I would like to take up this question of the functioning of the hydropic cells with respect to the β-cells, because in the cat diabetes persisting for some time after treatment with growth hormone may show spontaneous remission with still a very severe hydropsis of the β-cells (1948, *Lancet*, *2*, 955). In one animal that showed spontaneous recovery of diabetes without treatment, Mr. K. C. Richardson found that there was, in the pancreas, no β-cell which was not intensely hydropic. Either one must assume that these hydropic β-cells were secreting insulin, or that the animal had somehow managed either to do without insulin or to do with very much less insulin than normally. My own view is that those intensely hydropic β-cells were secreting insulin. The fact that hydropsis is often associated with an accumulation of glycogen in the β-cells may suggest that it is not such a pathological change as one thought at one time.

Ferner: That is quite right. The hydropic β-cells after treatment with other hormones undergo normal degranulation, but the hydropsis of the α-cells after IPTD or synthalin is a dissolution of their contents *in loco*, and there are no signs of degranulation. This is, in my opinion, a pathological behaviour.

Best: We now see complete degranulation of β-cells with no extractable insulin, and degranulation with lots of insulin. In the obese hyperglycaemic mice there is degranulation of the β-cells but the insulin content is well above the normal level. This has been shown by Wrenshall and Meyer.

Mosca: With regard to degranulation of the islet cells, we have performed some experiments on fish pancreas, which are only islets without exocrine tissue. In some fish, indeed, the two tissues are separate. This provides good material for endocrinological purposes, both from the morphological and biochemical points of view. We have noticed (Mosca, L. (1955), *Biol. latina*, *8*, 1) complete degranulation of the α-cells with very sharp blood sugar rise in fish after anoxia. This hyperglycaemia after anoxia in fish is a well known phenomenon (McCormick, N. A., and

Macleod, J. J. R. (1925), *Proc. roy Soc.*, B, 98, 1). The α-cells apparently recover after some time. This means that this phenomenon can be present in α-cells under certain normal functional conditions.

Young: You would argue from the fish to other animals?

Mosca: Perhaps in other animals not all the elements, but some of them, can be degranulated though still keeping their function.

Goldner: Dr. Mosca, is it correct that the extract of the argyrophil [illegible]

METABOLIC EFFECTS OF α-CELL DESTRUCTION

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THE history of the discovery of the α-cell hormone seems to be confusing. It is very closely connected with the names of those research teams which, since the discovery of insulin by Banting and Best, have tried to throw light on the pathophysiology of diabetes. We all know the merits of Murlin, Bürger, Sutherland, de Duve, Cori, Young, Foà, Goldner, Staub, Sinn, Behrens, Thorogood, Candela, Cavallero and many others, who contributed important new material to the essence of the rôle which glucagon plays in metabolism. Now we have tried, by means of studying the metabolic changes which take place after destruction of the α-cells, to confirm and extend those effects of HGF which up to now have been proved or discussed. Following on the discovery by Davis (1952) of the α-cell-damaging properties of decamethylenediguanidine (synthalin A), we have tried to produce a photographic negative, as it were, of the function of the α-cells, by observation of deficiency symptoms arising after destruction of these cells. In doing this we hoped to mark the region of effects of the α-cell hormone. In the course of these experiments we found that the blood-sugar level lowering property of *p*-aminobenzenesulphonamido*iso*propylthiodiazole (IPTD), described by Janbon, Lazergues and Métropolitanski (1943), is caused by an α-cell toxicity (v. Holt *et al.*, 1954, 1955*a*). Prof. Ferner has just shown you the morphology of cell damage and disappearance (v. Holt and Ferner, 1955). In the course of α-cell destruction by synthalin or IPTD, rabbits as well as rats developed a severe hypoglycaemia and often fatal shock unless glucose was given.

Twenty to twenty-four hours after the injection of synthalin we found a total loss of glucagon in the pancreas of rats, which showed severe hypoglycaemia and shock, similar to those rats in which we found widespread damage of α-cells (Table I).

Table I

GLUCAGON CONTENT OF RAT PANCREAS 20–24 HOURS AFTER INJECTION OF SYNTHALIN (15 MG./KG. INTRAPERITONEALLY), OR IPTD (1 G./KG. INTRAPERITONEALLY). $P < 0\cdot05$

	Glucagon content (µg./rat)	*Number of glands used for the extraction*	*Number of test rats*
Normal rats	$2\cdot4^{+1\cdot5}_{-1\cdot3}$	25	47
Synthalin-treated rats	$0\cdot0 + 0\cdot1$	15	24
IPTD-treated rats	$0\cdot4^{+1\cdot4}_{-0\cdot4}$	10	21

In the case of IPTD-treated animals there was a marked diminution of glucagon. The difference in the glucagon content perhaps finds its explanation in the fact that the morphological appearance of α-cells and the stage of cell damage in IPTD-treated animals at the time of sacrifice are not so uniform as in synthalin-treated rats. The possibility must also be discussed that in IPTD-treated rabbits the hormone remains in an active extractable form in the broken cells. The latter might explain why Prof. de Duve, who was so kind as to test the pancreatic glucagon content of IPTD- [illegible]

sugar test in cortisone-treated adrenalectomized rats. It is possible to determine HGF concentrations in a range up to $0\cdot25$ µg. glucagon in $0\cdot1$ ml (Fig. 1). A constant régime of

METABOLIC EFFECTS OF α-CELL DESTRUCTION

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Table II

HISTOLOGICAL APPEARANCE OF ISLETS IN RABBITS AND BLOOD SUGAR FLUCTUATIONS AFTER ORAL INGESTION OF DIFFERENT 1 : 3 : 4-THIODIAZOLE DERIVATIVES

Substance	*Dose g./kg*	*Maximum blood sugar change as percentage of fasting blood sugar*	*Histological appearance 24 hours after start* α-*cells*	β-*cells*	*Number of rabbits*
p-Aminobenzene-sulphonamide-5-n-propyl-1 : 3 : 4-thiodiazole	1·0	+ 0 − 72 (12) shock	1* 3†	4‡	73
	1·0	+ 0 − 53 (12)	2*	1‡	70
p-Aminobenzene-sulphonamide-5-isobutyl-1 : 3 : 4-thiodiazole	1·0	+ 0 − 56 (3)	2†	§	74
	1·0	+ 0 − 38 (5)	1*	§	76
p-Aminobenzene-sulphonamide-5-diethylmethyl-1 : 3 : 4-thiodiazole	1·0	+ 0 − 34 (21)	2*	2‡	313
	1·0	+ 0 − 33 (24)	2*	2‡	314
p-Aminobenzene-sulphonamide-5-hexyl-1 : 3 : 4-thiodiazole	1·0	+ 0 − 46 (9)	1*	1‡	301
	1·0	+ 0 − 72 (22)	1*	2‡	302
p-Aminobenzene-sulphonamide-5-heptyl-1 : 3 : 4-thiodiazole	1·0	− 6 (1) − 19 (13)	(*)	(‡)	56
	1·0	+ 0 − 24 (7)	§	§	77
2-Amino-5-isopropyl-1 : 3 : 4-thiodiazole	0·5	+ 0 − 31 (10)	1*	1‡	311
	1·0	+ 211 (3) − 15 (13)	3*	2‡	312
2-Acetylamino-5-sulphonamide-1 : 3 . 4-thiodiazole	0·5	+ 41 (2) − 0	1* 1†	2‡	69
	0·5	+ 39 (2) − 0	1* 1†	2‡	72

* Stage of increase in size and decrease in number of the α-granules.
† Stage of fragmentation and disappearance of α-cells.
‡ Granular demarcation of β-cell edges
§ No histological changes
*, † and ‡ in 4 degrees.

Figures in brackets = number of hours after oral ingestion.

food and cortisone treatment is necessary to achieve good results.

The histological localization of the cell damage as well as the disturbance of the hormone production, together with the finding that a lowering of the blood sugar level in the pancreatectomized animal can be produced neither by synthalin (Rathery, Kourilsky and Gibert, 1928; Ralli and Tiber, 1929) nor by IPTD (Loubatières, 1944), localize the essential action

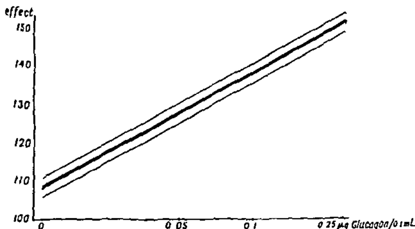


FIG 1. HGF dose-response line in adrenalectomized, cortisone-treated rats. Abscissa: arc tg-transformation of µg HGF (Eli Lilly lot no. 208–158 B–197)/0 1ml.
Ordinate: $\frac{\text{max. blood sugar increase}}{\text{initial blood sugar value}} \times 100$.

of these drugs in the σ-cells of the islets of Langerhans. Therefore I think we have good reason to connect the observed metabolic changes with the loss of hormone production and the ensuing disturbance of the hormonal equilibrium. Further investigations seem to show that other compounds also which have a thiodiazole ring in their molecule show a blood-sugar lowering activity and an α-cell destroying property (Table II). As to the β-cells, only signs of functional irritation are seen. The upsetting of the hormonal balance after the loss of function of the α-cells causes, besides the hypoglycaemia, a rapid

this initial blood sugar fluctuation is not attributable to a liberation of glucagon from the decaying α-cells.

The metabolic changes observed after α-cell destruction stress the essential importance of glucagon in maintaining a normal blood sugar level and liver glycogen content in close co-operation with insulin. Therefore it seemed to be of great interest to examine whether glucagon, whose action was not balanced by insulin, is partly responsible for the metabolic

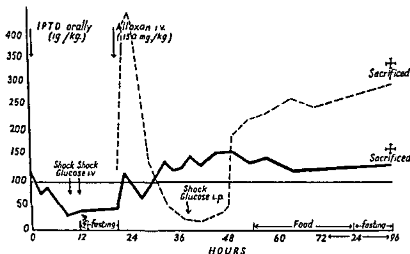


FIG. 2. Blood sugar course after alloxan injection into an IPTD-treated rabbit (——) in comparison with a normal one (— — —).

appearance of alloxan diabetes. The latter is a metabolic situation which is morphologically characterized by a change in the α : β-cell ratio with a perfect predominance of α-cells (Ferner, 1953). If the unbalanced action of HGF were one of the causes of alloxan diabetes, the preceding destruction of the α-cells must inhibit the development of a typical alloxan diabetes, and on the other hand an isolated loss of the α-cells in alloxan diabetic animals can be expected to restore the metabolic balance. We have shown that this is the case (v. Holt *et al.*, 1954, 1955*b*). Pretreatment with IPTD, in the rabbit, inhibits the development of alloxan diabetes in

depletion of liver glycogen (Table III), which may be the result of the unbalanced action of insulin. The latter causes similar changes, according to the results of Cori (1925) and Swenson (1945). The glucose produced by the glycogenolysis is quickly metabolized, perhaps by the action of insulin. This is indicated by the increase in the level of citric acid in the blood (Mârtensson, 1951), the decrease in easily hydrolysable phosphorus, as well as by the decrease in the fraction of hexoses and hexosephosphoric esters in the liver and the unaffected extrahepatic glycogen level (Table III). Experiments with adrenalectomized rats exclude a *sudden secretion of epinephrine* by the suprarenal medulla as the cause of liver glycogen depletion (v. Holt, v. Holt and Kröner, 1955). The initial hyperglycaemia often observed after IPTD injection cannot be found in the adrenalectomized animal. Therefore

Table III

Some Metabolic Changes after Synthalin
(Mean Values of (X) Animals)

	3 hours after intraperitoneal injection of 15 mg. synthalin/kg.	Control rats	Significance
Extrahepatic glycogen mg./100 g. rat	32 9 (10)	32 2 (10)	—
Liver glycogen g./100 g. dry liver	0 11 (26)	21·6 (21)	$P < 0{\cdot}001$
Hexoses and hex. phosphor. esters mg./100 g dry liver	18 0 (6)	283·0 (3)	$P < 0{\cdot}001$
7 min. P mg./100 g. dry liver	50 5 (6)	72 6 (3)	$P < 0$ 05
Inorg. P mg./100 g dry liver	117 5 (6)	83·0 (3)	$P > 0{\cdot}05$

on the other hand HGF injections repeated over a long period increase the liver glycogen level (Root, 1954), similar to the glycosteroid action, our results support the view, already held by Vuylsteke, Cornelis and de Duve (1950), that some synergistic relation exists between the function of the α-cell system and the adrenals.

Table IV

METABOLIC PATTERN IN RATS DURING THE ALLOXAN DIABETIC AND 'ISLETLESS' STAGE.

Number of Rats	*Glycosuria g./day* before synthalin	after synthalin	*Urinary nitrogen mg./day* before synthalin	after synthalin	*Diuresis ml./day* before synthalin	after synthalin	*Number of α cell groups/ 100 mm².*
190	1·50	0·23	320	212	97	19	1
192	2·71	0·45	470	158	50	28	11
204	0·17	1·90	360	110	100	42	12
217	7·10	0·84	279	175	90	14	8
218	5·45	0·03	171	100	62	7	-
317	2·42	0·01	144	18	8	0	12
321	3·06	0·10	230	175	40	17	
333	3·63	0·57	246	136	86	5	5
344	9·41	0·42	150	131	84	5	3

Liver Glycogen content g./100 g. dry liver

Normal	Alloxan diabetic	'Isletless'
0·67 (mean value of 11 rats)	0·77 (mean value of 6 rats) mean value of α-cell groups/100 mm.² 80	0·85 (mean value of 12 rats)

Experiments concerning the action of STH in isletless animals ought to throw light on the question of whether at least a part of the diabetogenic action of growth hormone is mediated by stimulation of HGF secretion. The results of Bornstein, Reid and Young (1951) and also of Foà and co-workers (1953) have revealed close connections between STH

of the loss of β-cells (Fig. 2). The difference in the behaviour of the blood sugar during the initial phase of the alloxan action may be explained by the decrease in liver glycogen due to the loss of α-cell function. The diabetic hyperglycaemia, too, returns to a normal level after treatment of the animals with IPTD or synthalin. Histological examination reveals a *total loss of both types of the islet cells. Due to the drastic* changes in metabolism which take place, only a small number of animals stay alive in the course of such experiments. Besides the isletless rats we obtained a greater number of *more or less unaffected diabetic animals.*

There was a close relationship between the decrease in liver glycogen content and the degree of α-cell loss. The correlation coefficient amounted to $r = 0 \cdot 54$, the measure of certainty ($\sqrt{r}$) to $B = 0 \cdot 33$. This means that 33 per cent of all causes which can diminish the liver glycogen content are explained by the loss of the α-cells ($P = 0 \cdot 01$). In regard to the urinary nitrogen there exists a similar correlation to the diminution of the α-cells. About 40 per cent of all causes which can be responsible for a decrease in urinary nitrogen are explained by the loss of α-cells ($r = -0 \cdot 631$, $B = 0 \cdot 398$, $P = 0 \cdot 01$). A direct correlation between the decrease in glycosuria and the reduction of the α-cell count was not significant, but in accordance with the good correlation between the decrease in urinary nitrogen and the glycosuria ($r = 0 \cdot 596$, $B = 0 \cdot 355$, $P = 0 \cdot 05$), the latter is influenced indirectly by the condition of the α-cell system. The decrease in urinary nitrogen in diabetic animals after the loss of the whole islet function makes it probable that the α-cell hormone is concerned with catabolic protein metabolic processes in a way which is not yet known. The increased liver glycogen content in diabetic animals is normalized, too, after α-cell destruction. These results (Table IV, Fig. 3) show that apparently the α-cell hormone plays an important rôle in the development of diabetic symptoms.

Since adrenalectomy causes a normalization of the alloxan diabetic liver glycogen content (Morita and Orten, 1950) and

HGF requirement of the organism due to the action of STH on the tissues, may cause the observed metabolic changes.

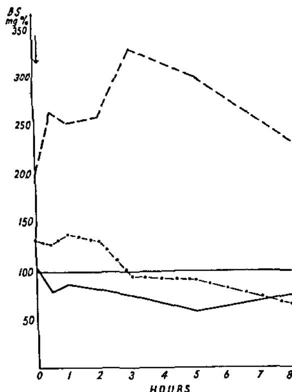


FIG. 4. Effect of STH (↓ = 3 mg STH/350 g rat i p.; Armour lot M 308) on the blood sugar of an isletless (-·-), an alloxan diabetic (— —), and a normal (——) rat (14 hours fasting).

The gain in body weight, however, shows that the growth-promoting activity of STH can be observed also in the absence of the α-cell system.

Although the elimination of both types of the islet cells results in a nearly balanced metabolic situation—as measured by estimations of blood sugar, excretion of glucose and nitrogen

and the α-cell system in other types of experiments. Our results obtained by STH-administration to isletless animals seem to indicate the importance of the presence of an intact α-cell system in the manifestation of the diabetogenic activity of growth hormone in rats. The hyperglycaemia observed

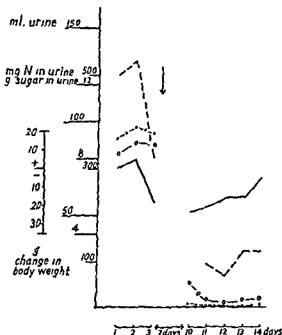


FIG. 3. Glycosuria (— —·—), urinary nitrogen (— — —), diuresis (o—o), and body weight (——) of an alloxan diabetic rat before and after injection of 12 mg synthalin/kg. i.p. (↓).

after STH-injections into alloxan diabetic rats does not take place in isletless animals (Fig. 4). The increase, too, in the glycosuria, which follows injection of STH into an alloxan diabetic rat, cannot be observed in an isletless animal (Fig. 5). In our opinion this does not prove an α-cell trophic function of STH, because also an indirect effect, mediated by an increased

metabolism. Whereas the classical experiment of Minkowski produces diabetes, the isolated destruction of the islets by

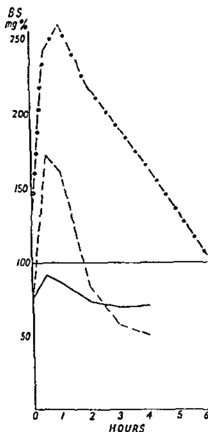


FIG 6. Sugar tolerance (1g. /kg. orally) in isletless rats (— —) compared with normal (——) and alloxan diabetic (o—o) rats

chemical means is followed by a normoglycaemic level but potentially diabetic condition, as shown by the impaired sugar tolerance.

and the level of liver glycogen—isletless rats are marked by a severe disturbance of the blood sugar regulation (Fig. 6). Though the glucose peak is very high in isletless animals, the blood sugar returns to a normal level within the same time as in healthy animals, contrary to the behaviour of diabetic rats.

We think that the latter result is important for the following reasons: firstly, it shows that an active regulation of the blood

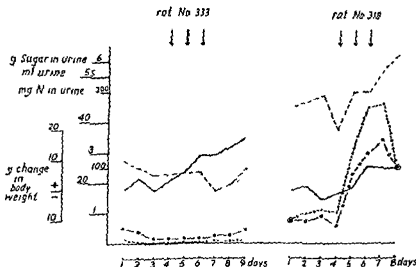


FIG. 5 Glycosuria (—), urinary nitrogen (— —), diuresis (o—o), and body weight (———) of an isletless rat (No 333) after injection of *STH* (↓ = 3 mg./350 g. s.c) in comparison with a synthalin-treated animal (No. 318), whose alloxan diabetes persisted

sugar level can be achieved in the absence of islet tissue; secondly, it proves the existence of an intact liver function because only the latter is able to effect such a regulation, according to the results of Soskin and Levine (1952). At the same time the importance of a functional co-operation of all parts of the pancreas is stressed, in so far as it is necessary that the internal secretion as well as the so-called external secretion bring about the balanced state of carbohydrate

clude that the islet cells are not essential for this part of the action of synthalin. Is that correct?

von Holt: I think that the results with synthalin in depancreatized animals are very different in the different laboratories (Rathery, F., Kourilsky, R., and Gibert, S. (1928), *C. R. Soc. Biol., Paris*, 99, 282; Ralli, E. P., and Tiber, A. M. (1929), *J. Pharmacol.*, 37, 451). There is no clear opinion about the action of synthalin in depancreatized animals.

de Duve: It should be recalled that the first investigation on the [illegible]

alternative possibility of a stimulation of the α-cells, which had not come into the foreground at that time. However, his results are of importance in showing that the pancreas is necessary for the action of IPTD.

von Holt: Loubatières measured the blood-sugar lowering activity in circulation experiments and found an increase in insulin-like activity in the blood of animals treated with IPTD. I think this is due to loss of the [illegible]

[illegible]

von Holt: We have never seen signs of damage in the adrenal cortex, but sometimes, especially in long-term experiments with IPTD, we observed an activation of the cortex.

Ferner: In other experiments we have seen an increased activity of the adrenal cortex.

Randle: Rose (1951, *Aust. J. exp. Biol. med. Sci.*, 29, 469) has obtained evidence suggesting that the adrenal cortex is overactive in alloxan diabetes. I just wondered if α-cytotoxic drugs might alleviate the diabetes by depressing adrenocortical activity.

Best: I do not know how the dosages of synthalin used in the experimental animals in the recent work compare with the amounts used clinically many years ago. There was no doubt about the injury to the liver in the clinical experiments—some of the livers were practically falling apart. There was no study of the effect of synthalin on α-cells at that time

Goldner: I should like to go back to Prof. von Holt's experiments in which he was unable to induce alloxan diabetes after treating the animals with synthalin and IPTD. I am not fully convinced that it is permissible to conclude that this phenomenon is due to the effect of these agents

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DISCUSSION

Young: Prof. von Holt, in your experiments in which you showed

von Holt: No, the food was given unconditioned, ad lib., in both groups

Young: Did the food intake rise under the influence of growth hormone?

von Holt: This was not measured.

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Concerning Dr. Goldner's last deduction, I think that it is not the rôle of the islets of Langerhans in the aetiology of diabetes which must be re-evaluated, but the limits of the contribution of the α-cell system to the development of alloxan diabetic hyperglycaemia which must be fixed.

Fod: What is the interval of time between the injection of the two materials? Is it possible that the α-cytotoxic material may destroy the alloxan in the circulation or *in vitro*? Also, it has been implied several times that the α-cells are the site of origin of glucagon. I would like to ask if this is a safe foundation on which to base the discussion of the next few papers.

von Holt: I think my answer to Dr. Goldner has answered your question also. The destruction of the α-cells proves that no inactivation of alloxan in the blood by IPTD has taken place. The interval between [illegible]

[illegible]

Metabolism, 3, 449)

Fig. 2 (p. 30) shows the blood sugar curves of fasting animals which were given intravenously aliquots of such extracts. The extract from the [illegible]

[illegible] from the α-cells, since the histological control demonstrated that the α-cells were destroyed. I have previously shown slides of the islets of [illegible]

[illegible] The α-cell damage by cobalt chloride is, however, not accompanied by loss of the hyperglycaemic factor from the extract. We have gone a step further In addition to the destruction of the islets, we have tried to destroy the exocrine portion of the pancreas by duct [illegible]

[illegible] and the α-cells can be considered as settled.

Young: You do think that your hyperglycaemic substance is glucagon?

[illegible]

lib
in

upon the α-cells. It is well known that a number of non-specific factors may interfere with the diabetogenic action of alloxan. There is also the as yet unexplained observation that clamping of the renal pedicles prevents alloxan diabetes, and just recently Dr. Jauregui of Puebla informed me that no diabetes will develop in the dog if the liver is temporarily eliminated from the circulation for 2–3 minutes after administration of alloxan. Could it not be that the effect on the liver which at least is established for synthalin has something to do with Prof. von Holt's findings? It would also be important to know in how many instances this inhibition of alloxan diabetes did occur, or conversely, *whether there were instances where alloxan diabetes developed in spite* of pretreatment with α-cytotoxic agents After all, the diabetogenic *dose of alloxan is defined as that amount of alloxan which will induce* diabetes in 80 per cent of the treated animals. There are always a number of animals which will not respond at all or which will die within the first 24 hours before diabetes becomes evident. I believe I remember from Prof. von Holt's published papers (1954, *Arch. exp. Path. Pharmak*, 229, 66, 78), that there were quite a few animals which died early and others which did develop diabetes If Prof. von Holt's observations are not due to non-specific factors or extrapancreatic effects of his agents

explained by a reflex arising from the manipulation on the pedicles.

think Dr Goldner himself has shown in earlier papers that the contact of

IPTD, histological examination of the islets revealed an insufficient destroying effect of IPTD on the α-cells. Furthermore, it could be shown that treatment with IPTD or synthalin in alloxan diabetic animals

Best: We have not done enough to make a decision on that point.

Behrens: Dr. Goldner, have you determined whether this hyperglycaemic material is sensitive to proteolytic enzymes?

Goldner: No.

Best: I wonder whether Prof. Sutherland or Prof. Cori has ever tried [illegible]

[illegible] ibits

[illegible]

Young: Have you shown in your experiments that ergotamine does not prevent the hyperglycaemic action of glucagon in diabetic animals?

C. F. Cori: We have not tried that.

Best: We have done that very carefully, testing with pure glucagon and with adrenaline, and you must satisfy the conditions that the glucagon is left intact and the adrenaline obliterated by the dose you use, before you go on to the other experiment

Lawrence: I did some clinical experiments many years ago which [illegible]

[illegible]

(1952, *Arch. int. Physiol*, 60, 128). Since then, we have treated a few more animals, using larger amounts of cobalt nitrate in order to achieve complete destruction of the α-cells. Preparations were obtained in which no intact α-cells could be seen and in those extracts no glucagon could be demonstrated

Goldner: I would like to make it quite clear: we do not doubt that [illegible]

[illegible]

hormone, and a blood sample was taken later from the pancreatic duodenal vein and simultaneously from the jugular vein; when these [illegible]

completely prevented in the diabetic animal when it has been treated with the adrenergic blocking agent, dihydroergotamine. These findings

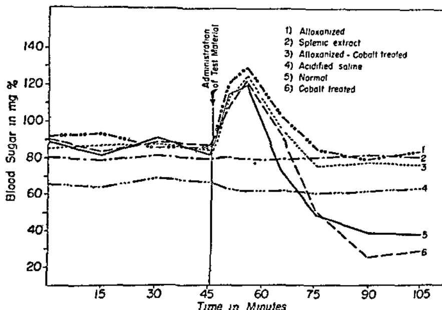


FIG. 2 (Goldner). Blood sugar response of anaesthetized fasting normal dogs to the intravenous injection of pancreatic extracts from cobalt-treated and/or alloxanized dogs, of canine spleen, and of acidified normal saline.

have stimulated us to begin a study of the liberation of adrenergic substances from the pancreas and other tissues after administration of growth hormone.

Fod: Was the ergotamine given to the donor animal or to the recipient or to both?

Best: Just to the recipient animal.

Randle: I should like to ask Prof. Best whether he considers that *dihydroergotamine is blocking the action of adrenergic* material released in the recipient dog after injection of the blood sample, or whether it is blocking the action of some adrenergic material present in the blood sample.

de Duve: Does this mean that the liver of an alloxan diabetic animal is [illegible]

[illegible]

experiments for many years. I wonder whether anyone has repeated them and whether there is any possible connection with the difference between the α: β-cell ratio of the pancreas of the young animal and that of the adult.

Goldner: A similar phenomenon has been seen in alloxan diabetes [illegible]

[illegible]

by Bürger to decrease with the progress of age [illegible]

[illegible]

under the other, but those findings have not yet been confirmed by other histologists. Indeed, some have failed to confirm the histological findings

Foà: I should like to ask another general question. We have heard of the possible occurrence of α-cell tumours; on the other hand, I. Mc- [illegible]

[illegible]

of α-cells?

von Holt. Yes, in the pediatric clinic in Hamburg there was a case of a child who died in severe hypoglycaemia (Bierich, J. R., and [illegible] 102, 49). [illegible] y altered [illegible] It is the

striking morphological pictures. The cytoplasm, which is normally very rich in colloid droplets, completely loses these droplets, and also loses all secretory granulations and becomes clumped and more intensely eosinophilic (Mosca, L. (1955), *Biol. latina*, 8, 1). From these islets it is impossible to extract any hyperglycaemic substance, while on the contrary, if we make up an extract from normal fish and inactivate insulin with potassium hydroxide, we get an enormous quantity of hyperglycaemic factor. (Malandra, B., and Mosca, L. (1952), *Rass. Fisiopat. clin. terap.*, 24/1, 43). This means that most probably this

made extracts from the whole pancreas, islets and exocrine tissue of fish. We have just u
no attempt to avoid
their fish. This has
of α-cell secretion, th
extracts.

Kerly: Some years ago, Dr. Audy carried out experiments in my laboratory on fish which came on ice from Plymouth and she did find glucagon in the islet tissue, which confirms your findings. It was assayed on liver slices.

do rabbits.

inhibitor of the supposed enzyme which destroys glucagon. assays on the extracts of cobalt-treated animals were done under these conditions, so that even small amounts of glucagon should have been detected.

Goldner: It seems that a normal parenchyma of the liver is essential for inactivation or destruction of glucagon. We have perfused a few livers of alloxanized frogs with glucagon and with commercial insulin. In these experiments the returning perfusate retained hyperglycaemic activity

PITUITARY GROWTH HORMONE AND BLOOD INSULIN ACTIVITY

P. J. RANDLE

Department of Biochemistry, University of Cambridge

I SHALL not attempt to review the indirect evidence which led to the belief that pituitary growth hormone exerts an influence upon the secretion of insulin by the β-cells of the pancreatic islets. The subject has been reviewed extensively by Young (1953)

Attempts to demonstrate, by direct means, an influence of growth hormone upon insulin secretion have been made by Anderson and Long (1947, 1948), Bornstein, Reid and Young (1951) and Foà and co-workers (1953). In these studies the blood sugar response of suitable test animals was utilized for the detection of insulin in blood plasma. The experiments of Bornstein and co-workers and of Foà and co-workers suggested that growth hormone promotes the secretion of an hyperglycaemic factor by the pancreatic islets of the rat, cat and dog, but failed to provide definite evidence for any influence of growth hormone upon the secretion of insulin by the pancreatic islets. Anderson and Long obtained evidence which suggested that growth hormone depressed the secretion of insulin by the isolated perfused rat pancreas when the level of glucose in the perfusing fluid was abnormally high. Young (1953) has suggested that the release of glucagon from the pancreatic islets under the influence of growth hormone might prevent the detection of insulin by the method which Anderson and Long used, and account for the apparent suppression of insulin secretion which they observed.

Groen and co-workers (1952) described a method of insulin assay based upon the glucose uptake of the isolated rat diaphragm. Since glucagon does not oppose the action of insulin upon the glucose uptake of the isolated rat diaphragm

von Holt: No

Goldner: Nor glycogen storage disease?

von Holt: No.

Behrens: Was there any reason to suspect that your islet-destroyed animals exhibited glycogen storage disease? You state that the glycogen is low, I think?

von Holt: Yes, the liver glycogen level after fasting corresponds to the normal level.

Experimental Design. In each experiment 4–6 hemidiaphragms were incubated in buffer, buffer + plasma (25 per cent plasma) and buffer + insulin (2 or 4 milliunits/ml.). The significance of any change in glucose uptake resulting from the addition, to the buffer, of plasma or insulin was established by the "*t*" test (Chambers, 1952). In experiments with rat plasma, the rats were studied in pairs in any one experiment so that direct comparison of plasma insulin activity between intact rats treated with growth hormone or saline; untreated intact and hypophysectomized rats; and hypophysectomized rats treated with growth hormone or saline, was possible. In this type of experiment, the significance of any difference between the mean glucose uptake of hemidiaphragms incubated in different plasma samples was established by the "*t*" test. In the majority of experiments with cat plasma, the cats were not studied in this way and the insulin activity of the plasma under different conditions was compared by reference to the same standard dose of insulin (4 milliunits/ml.) incorporated in each experiment. In this type of experiment, the significance of any difference between the mean glucose uptake of rat hemidiaphragms incubated in plasma + buffer, or insulin + buffer, was established by the "*t*" test.

An approximate value for the insulin activity of the plasma calculated as milliunits insulin/ml. of plasma was also derived in each case (1 milliunit = 10^{-3} unit). The insulin activity of the plasma was calculated from a log. dose (of insulin/response curve previously determined, the standard dose of insulin incorporated in each experiment being used to locate the position of the log. dose/response curve.

Results

Response of the isolated rat diaphragm to insulin

The influence of different concentrations of insulin upon the glucose uptake of the isolated diaphragm has been studied on eighteen different occasions. The regression of response

(Smith, 1953; Randle, unpublished experiments) it was of interest to reinvestigate the influence of growth hormone treatment upon the insulin activity of blood plasma, using for the detection of insulin activity in plasma the glucose uptake of the isolated rat diaphragm (Young, 1953).

Methods and Procedure

Growth Hormone. Pituitary growth hormone was prepared from ox pituitary anterior lobes by a modification of the method of Wilhelmi, Fishman and Russell (1948). Solutions for injection were prepared by dissolving the hormone in 1 per cent saline containing 1 : 10,000 merthiolate.

Animals. Intact cats (male or castrate) were fed unlimited amounts of a meat diet (Young, 1945) and water, and injected subcutaneously with growth hormone or saline each morning. Depancreatized cats received raw pancreas or pancreatin in addition to the meat diet, and their diabetes was controlled by subcutaneous injections of soluble insulin each morning and evening.

Intact and hypophysectomized rats (female albino wistar rats of 150–200 g.) were fed unlimited amounts of rat cake and water (Parkes, 1946). Growth hormone or saline was injected intraperitoneally, once each day to intact rats, and twice each day to hypophysectomized rats.

Collection of blood. Rat blood was taken from the inferior vena cava and cat blood from the exposed femoral vein, under evipan anaesthesia. Human blood was drawn from an antecubital vein. In each case a little heparin was added to prevent clotting and the plasma separated by centrifugation. Unless otherwise stated, rats and cats were taken from food and injected with growth hormone, saline or insulin 80 minutes before blood was collected. Human blood was collected 2–2·5 hours after the oral administration of 50 g. glucose.

Detection and estimation of plasma insulin activity

Experimental Procedure. The procedure described elsewhere (Randle, 1954c) was followed exactly.

and thawed). Insulin activity was detected in plasma from four totally depancreatized cats treated with insulin and the mean plasma insulin activity was significantly less than 16 milliunits/ml. When growth hormone was administered to three totally depancreatized cats treated with insulin, without

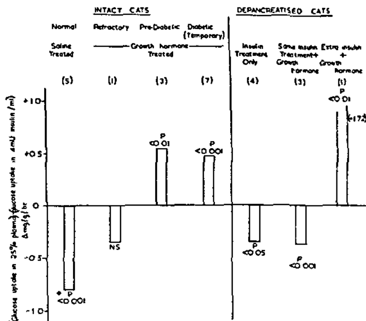


FIG 1. Insulin activity of plasma from intact and depancreatized cats Probability that difference between glucose uptake in buffer + insulin (4 mU/ml.) and buffer + plasma (25 per cent plasma) is due to chance. NS means $P > 0{\cdot}05$.
For details of treatment see Table I or text

alteration in the daily dose of insulin, the mean insulin activity of the plasma was still significantly less than 16 milliunits/ml. When a totally depancreatized cat, receiving insulin, was treated with growth hormone and the daily dose of insulin increased during the period of growth hormone treatment, the insulin activity of the plasma was significantly greater than 16 milliunits/ml. The insulin activity of plasma from

upon dose was significant on each *occasion and the relationship* between $\sqrt[3]{}$(glucose uptake by the diaphragm) and log. concentration of insulin in the suspending fluid did not depart from linearity in any instance. The index of precision was of the order of 0·35 and the mean slope 0·7439.

Earlier experiments suggested that the slope of the log. dose/response curve was constant (Randle, 1954*c*, 1955), but more recently significant variations in slope have been demonstrated (range of slopes $\pm$ st. error $= 0{\cdot}5966 \pm 0{\cdot}1080$ to $1{\cdot}1661 \pm 0{\cdot}1261$). The estimates of plasma insulin activity obtained by a single standard method (Randle, 1954*c*) are therefore only approximate estimates.

Influence of growth hormone on plasma insulin activity

The insulin activity of plasma from five intact cats treated with saline was, in every instance, significantly less than 16 milliunits/ml. A single intravenous injection of growth hormone did not alter the insulin activity of plasma from intact cats within five minutes of the injection. When intact cats were treated with growth hormone for several days the insulin activity of the plasma was increased, irrespective of whether the animals were prediabetic (plasma sugar less than 150 mg./100 ml.) or temporarily diabetic (plasma sugar greater than 150 mg./100 ml. with glycosuria which ceased within three days of stopping growth hormone treatment). The mean plasma insulin activity for both prediabetic and temporarily diabetic cats was significantly greater than 16 milliunits/ml. No insulin activity could be detected in fresh plasma from a cat which developed permanent diabetes after prolonged treatment with growth hormone, but insulin activity, *significantly less than 16 milliunits/ml., could* be detected in the same plasma samples after they had been repeatedly frozen and thawed.

Insulin activity could not be detected in plasma from three totally depancreatized cats deprived of insulin for 36–48 hours (fresh plasma or plasma which had been repeatedly frozen

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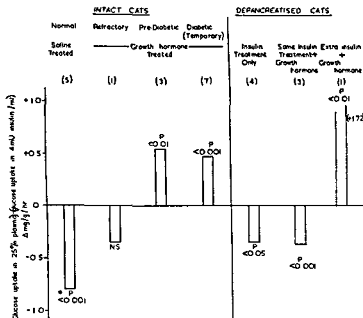


FIG. 1. Insulin activity of plasma from intact and depancreatized cats. Probability that difference between glucose uptake in buffer + insulin (4 mU/ml) and buffer + plasma (25 per cent plasma) is due to chance. NS means $P > 0 \cdot 05$.
For details of treatment see Table I or text

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Insulin activity could not be detected in plasma from three totally depancreatized cats deprived of insulin for 36–48 hours (fresh plasma or plasma which had been repeatedly frozen

Table I

INSULIN ACTIVITY OF PLASMA FROM NORMAL AND DIABETIC CATS

Type of cat and number studied	*State*	*Injected with*	*Total dose*	*Injection period (days)*	*Duration of diabetes (days)*	*Mean plasma insulin activity (milliunits insulin/ml.)*
Intact						
(8)	Non-diabetic	Saline	5–8 ml.	5–8	—	3·9
(3)	Pre-diabetic	Growth hormone	30–75 mg.	5–8	—	46
(6)	Idio-hypophyseal diabetic	Growth hormone	30–75 mg	6–8	0–2	56
(1)	*Meta-hypophyseal diabetic	Growth hormone	700 mg.	35	49	1·1
Depancreatized						
(3)	†Diabetic (no insulin)	—	—	—	3–35	None detected
(4)	‡Diabetic	Insulin	13-17U/day	—	—	7·6
(3)	‡Diabetic	Insulin and growth hormone	13-17U/day 25–60 mg.	4–7	—	7·3
(1)	§Diabetic	Insulin and growth hormone	28U/day 60 mg.	7	—	700

* Plasma collected 49 days after last injection of growth hormone. No insulin activity detected in fresh plasma. Estimate of insulin activity given for plasma which had been repeatedly frozen and thawed in solid carbon dioxide/acetone and water at 37° C

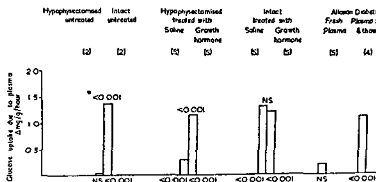
† Depancreatized animals deprived of insulin for 36–48 hours No insulin activity detected in either fresh plasma or plasma repeatedly frozen and thawed.

‡ Last injection of insulin was 8–10 units

§ Last injection of insulin was 40 units

intact cats treated with growth hormone or depancreatized cats treated with insulin or insulin + growth hormone was not altered when the plasma was repeatedly frozen and thawed. These results are illustrated in Fig. 1 and Tables I and II.

The insulin activity of plasma from five intact rats treated with growth hormone did not differ significantly from that of five saline-treated controls, though the growth-hormone-treated rats gained more weight than the saline-treated controls. In the rat hypophysectomy was followed, always,



by marked and significant reduction in the insulin activity of the plasma. This change, following hypophysectomy, could be prevented by growth hormone treatment, provided it was begun immediately after hypophysectomy and the hormone given twice daily (by intraperitoneal injection). By contrast, growth hormone added *in vitro* to the plasma of hypophysectomized rats did not restore the insulin activity to the plasma. No insulin activity could be detected in fresh plasma from *alloxan diabetic rats, but insulin activity* could be detected in the same samples of plasma after they had been frozen and thawed repeatedly. These results are illustrated in Fig. 2 and Tables III and IV.

Table III

INSULIN ACTIVITY OF PLASMA FROM NORMAL, HYPOPHYSECTOMIZED AND ALLOXAN DIABETIC RATS

Type of rat and number studied	*Injected with*	*Injection period (days)*	*Total dose*	*Interval after hypophysectomy (days)*	*Mean weight change (grams)*	*Mean plasma insulin activity (milliunits insulin/ml.)*
Intact						
(5)	Saline	2–18	1–8 ml.	—	+ 11	11
(6)	Growth hormone	2–18	2–19 mg	—	+ 26	15
Hypophysectomized						
(6)	Saline	2–5	2–5 ml.	2–5	− 8	None detected
(6)	Growth hormone	2–5	2–5 mg.	2–5	+ 1 3	11
Alloxan diabetic						
Fresh plasma (7)	—	—	—	—	—	None detected
a Frozen plasma (4)	—	—	—	—	—	7 6

a Plasma frozen and thawed repeatedly in solid carbon dioxide/acetone and water at 37°C

Numbers in brackets = number of animals studied.

Table II

THE INFLUENCE OF A SINGLE INTRAVENOUS INJECTION OF GROWTH HORMONE UPON THE INSULIN ACTIVITY OF PLASMA COLLECTED FROM INTACT CATS

Cat number	*Plasma collected*	*Glucose uptake mg./g. of wet diaphragm/hr. Mean ± S.E. of mean*		*t*	*P*
		In buffer alone	*In buffer + plasma (25% plasma)*		
269	Before growth hormone	a 1 74 ± 0 03 (4)	b 3 12 ± 0·33 (5)	b − a, 3·12 c − a, 4·49	< 0 02 < 0·01
	*After growth hormone		c 2·89 ± 0·18 (5)	b − c, 0 52	> 0 6
277	Before growth hormone	a 2 37 ± 0 04 (4)	b 2 83 ± 0·14 (5)	b − a, 2·40 c − a, 2·86	< 0·05 < 0·02
	*After growth hormone		2·74 ± 0 09 (5)	c − b, 0·46	> 0 6

* Plasma collected five minutes after the intravenous injection of 2·5 mg of growth hormone.
Numbers in brackets = number of animals studied.

The insulin activity of plasma from many acromegalic patients was found to be greater than that of plasma from normal people (Randle, 1954*a*) and the insulin activity of plasma from some cases of panhypopituitarism to be less than that of plasma from normal people (Randle, 1954*b*).

Discussion

Relation between plasma insulin activity and plasma insulin content

The insulin activity of blood plasma in any bioassay for insulin results from the mean effect of factors in the plasma which, in the bioassay, have insulin-like or anti-insulin actions (Randle, 1955). The insulin-like action of plasma from normal animals upon the glucose uptake of rat diaphragm would appear to depend upon the presence of insulin in the plasma; since cysteine treatment (which inactivates insulin—du Vigneaud *et al.*, 1931) abolishes the insulin activity of normal human plasma (Groen *et al.*, 1952; Vallance-Owen and Hurlock, 1954; Randle, 1954*c*); and after total pancreatectomy in the dog (Groen *et al.*, 1952) or cat (Table I) insulin activity cannot be detected in the plasma unless the animal is treated with insulin. Six-point assays with plasma from nine normal fasted rats have shown that the regression of response upon dose is not the same for plasma as for insulin (Randle, unpublished experiments). Thus although the insulin activity of blood plasma, as estimated by the glucose uptake of the rat diaphragm, is dependent upon the presence of insulin in the plasma, it does not necessarily provide an estimate of insulin alone in the plasma.

Bornstein and Park (1953) have suggested that the insulin-like action of normal rat plasma upon the glucose uptake of rat diaphragm may result from a non-specific effect of plasma protein. This seems unlikely since plasma from hypophysectomized rats and from depancreatized cats and dogs deprived of insulin, unlike that from normal animals of the same

Table IV

INFLUENCE OF GROWTH HORMONE *in vivo* OR ADDED *in vitro* UPON THE INSULIN ACTIVITY OF PLASMA FROM HYPOPHYSECTOMIZED RATS

	Addition to plasma in vitro *of*	*Glucose uptake mg./g. of wet diaphragm/hr. Mean ± S.E. of mean*	
		In buffer alone	*In buffer + plasma (25% plasma)*
Plasma from four hypophysectomized rats injected with saline, 0 5 ml, b.d for two days after operation	No addition		*b* 2·39 ± 0·10 (10)
	Growth hormone added, 40μg./ml.	*a* 2·16 + 0 04 (8)	*c* 2 19 ± 0 13 (10)
Plasma from two hypophysectomized rats injected with growth hormone, 0·5 mg, b d. for two days after operation	No addition		*d* 3 15 ± 0·11 (10)

t for *b* − *a* is 1·94, $P > 0\ 05$. *t* for *c* − *a* is 0·2, $P > 0{\cdot}8$
t for *d* − *a* is 7·09, $P < 0{\cdot}001$. *t* for *c* − *b* is 1·18, $P > 0\ 2$
t for *d* − *b* is 5·00, $P < 0{\cdot}001$. *t* for *d* − *c* is 5·69, $P < 0\ 001$

Number in brackets = number of animals studied

and this suggests the possibility that growth hormone does not alter the amount of insulin in the plasma of the intact rat.

Insulin activity appeared in plasma from alloxan diabetic rats and metahypophyseal diabetic cats after the plasma had been repeatedly frozen and thawed. Freezing and thawing would be expected to inactivate an inhibitor of the type described by Bornstein and Park (1953) and Bornstein (1953). These results suggest that some insulin was present, though in a masked form, in the plasma of alloxan diabetic rats and metahypophyseal diabetic cats. This finding is consistent with the observation that these forms of diabetes may remit spontaneously.

Relation between plasma insulin content and insulin secretion

The insulin content of blood plasma is influenced both by the rate at which insulin is secreted and by the rate at which it is utilized or destroyed by the tissues. Changes in plasma insulin content may reflect alterations in the rate of either or both of these processes. Since insulin has been extracted only from the pancreas (Best, Jephcott and Scott, 1932), it would appear that insulin secretion is an exclusive function of the pancreas. Two pathways for the utilization of insulin have been described. The extensive investigations of Stadie and his associates (Stadie, 1951, 1954) would appear to show that insulin is removed from the blood plasma and bound irreversibly, by those tissues on which it acts (see also Ottaway, 1954; Chayen and Smith, 1954). The experiments of Mirsky and his associates suggest that there is, in addition, an enzyme system in many tissues, which inactivates and degrades insulin (Mirsky, 1953).

The observation that growth hormone treatment does not enhance the insulin activity of plasma from insulin-treated depancreatized cats unless the dose of insulin is increased during the period of growth hormone treatment suggests that growth hormone treatment does not reduce the rate at which

species, has little or no influence upon the glucose uptake of rat diaphragm.

The influence of growth hormone on plasma insulin activity

Pituitary growth hormone has no influence upon the glucose uptake of the isolated rat diaphragm, under the conditions of *these experiments, when added in vitro to the suspending* fluid (Randle, 1954*d*). This confirms the earlier observations of Park and co-workers (1952), but not those of Bulbrook and Ottaway (1955), who claim that growth hormone can either stimulate or depress the glucose uptake of rat diaphragm. When both growth hormone and insulin are added together to the suspending fluid, under the conditions of these experi*ments, growth hormone can enhance the action of insulin* upon the glucose uptake of rat diaphragm (Randle, 1954*d*). Nevertheless, growth hormone does not alter the insulin activity of plasma from normal people (Randle, 1955) or hypophysectomized rats (Table III) when added *in vitro* to the plasma, nor does a single intravenous injection of growth hormone have any influence upon the insulin activity of *plasma from intact cats within five minutes of the injection* (Table II). The enhanced insulin activity of plasma from intact cats and hypophysectomized rats treated with growth hormone and from acromegalic patients must therefore depend upon a change in the level of some factor whose presence in the plasma is conditioned by growth hormone. In the intact cat, this factor may be identified with insulin, since growth hormone treatment does not enhance the insulin activity of plasma from depancreatized cats receiving insulin unless the dose of insulin is increased during the period of growth hormone treatment. In the rat the reduction in plasma insulin activity *which follows hypophysectomy, and which may be* prevented by growth hormone treatment, may also reflect changes *in the insulin content of the plasma, but evidence in* support of this is lacking. Growth hormone treatment does not influence the insulin activity of plasma from intact rats

secretion by the pancreatic islets of the cat, and suggest, also, that a temporary form of diabetes may appear in the cat as a consequence of growth hormone treatment at a time when the plasma level of insulin is still elevated. The enhanced insulin activity of plasma from acromegalic patients may also reflect an enhanced rate of insulin secretion by human pancreatic islets in response to hypersecretion of growth hormone in acromegaly. Bennett, by means of the pancreatic transplant technique, obtained evidence that in the dog, too, growth hormone promotes an enhanced rate of insulin secretion (Bennett, 1955). Nevertheless, it should be emphasized that there still lacks a direct and unequivocal demonstration that growth hormone influences, directly or indirectly, the secretion of insulin by the pancreatic islets of any species.

Studies of the influence of insulin and growth hormone treatment upon glycogen deposition *in vivo* in the adipose tissue of intact rats led Scott and Engel to conclude that growth hormone did not increase the rate of insulin secretion by the pancreatic islets of the rat (Scott and Engel, 1950). Likewise, Anderson and Long (1947, 1948) concluded from studies with the isolated perfused pancreas of normal fasted rats that growth hormone did not enhance the rate of insulin secretion by the pancreatic islets of the rat. Estimations of plasma insulin activity by means of the glucose uptake of the isolated rat diaphragm in the present investigation have failed to provide evidence for any influence of growth hormone treatment upon the rate of insulin secretion by the pancreatic islets of the intact rat.

The evidence at present available suggests that growth hormone promotes an enhanced rate of insulin secretion by the pancreatic islets of the cat, dog and perhaps man, but not by those of the intact rat. There may thus be a correlation between the diabetogenicity of growth hormone in different species and its ability to promote an enhanced rate of insulin secretion; but such a possibility requires substantiation by studies in further species, and in animals of the same species at different ages.

insulin is utilized by the tissues of the cat. The rise in the insulin content of the plasma which accompanies growth hormone treatment in the intact cat may therefore reflect an increase in the rate of insulin secretion by the pancreatic islets.

If the fall in plasma insulin activity which follows hypophysectomy in the rat is due to a reduction in the insulin content of the plasma, then hypophysectomy is followed by either a diminished rate of insulin secretion or an increased rate of insulin utilization, or by a combination of both processes. Similarly, the action of growth hormone in preventing the fall in plasma insulin activity which normally follows hypophysectomy in the rat may be mediated through the mechanisms of insulin secretion or insulin utilization. These two possibilities cannot at present be distinguished, though the work of Stadie and his associates has suggested that the diaphragm muscle of hypophysectomized rats may bind more insulin than that of normal rats and that this difference is annulled when hypophysectomized rats are treated with growth hormone and cortisone. The work of Stadie and his associates has suggested also that insulin binding by rat diaphragm muscle may be severely reduced in alloxan diabetes, and this change could account for the presence of insulin activity in the plasma of alloxan diabetic rats, since it is generally accepted that little insulin is secreted by the pancreatic islets of alloxan diabetic rats (see, for example, Anderson and Long, 1948).

The influence of growth hormone on insulin secretion

Studies of nitrogen retention in intact, depancreatized and depancreatized-hypophysectomized cats under the influence of growth hormone treatment led Milman, de Moor and Lukens to conclude that growth hormone provokes the secretion of extra insulin by the pancreatic islets of the cat (Milman, de Moor and Lukens, 1951). Estimations of plasma insulin activity by means of the glucose uptake of the isolated rat diaphragm support the concept that growth hormone promotes, directly or indirectly, an enhanced rate of insulin

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DISCUSSION

R.-Candela: We have studied the plasma insulin effect in a case of acromegaly and found a very high insulin activity of the plasma, confirming Dr. Randle's theory.

Randle: This slide illustrates the enhanced insulin activity of acro-

blood samples were collected 4½ hours after the morning dose of insulin,

TABLE I (*Randle*)

THE INFLUENCE OF PLASMA FROM NORMAL AND ACROMEGALIC PEOPLE UPON THE GLUCOSE UPTAKE OF THE ISOLATED RAT DIAPHRAGM

Sample No.	*Source of plasma*	*Plasma sugar mg /100 ml.*	*Glucose uptake mg./g /hr. Mean ± S.E. of mean*
1a	Normal person	70	2·84 ± 0·15 (4)
1b	Diabetic (not acromegalic) receiving insulin	305	2·85 ± 0·21 (4)
1c	Diabetic acromegalic receiving insulin	360	4·00 ± 0·21 (4)
2a	Normal person	80	2·60 ± 0·13 (8)
2b	Diabetic acromegalic receiving insulin	190	3·62 ± 0·09 (8)

For (1c–1b) and (1c–1a) $P<0{\cdot}01>0{\cdot}001$ For (2b–2a) $P<0{\cdot}001$
Figure in parentheses is number of observations.

Acknowledgements

I wish to thank the Medical Research Council for personal and expenses grants in support of my work on plasma insulin activity; Professor F. G Young for much helpful advice and criticism during the course of this work and the preparation of this communication; and Mr M. J. Brittain and Miss D. Cope for technical assistance.

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Ltd.

Young: What do people here who have had experience of this type of work feel about this question? Is the evidence that growth hormone stimulates the secretion of insulin satisfactory?

evidence—direct and indirect—to indicate secretion of insulin by growth hormone.

Young: The species differences provide a complication that had not been realized before. Evidence against the view has been obtained in rats, which now are found not to show the same response as cats.

Lawrence: I would like to ask a simple clinical question: I suppose that by now you have done a good many diabetics, and I wonder what you found the insulin content of their plasma to be?

Randle: All the diabetic patients that I have studied, with the exception of those in severe diabetic ketosis, have insulin activity in the plasma. These include examples of both of the major clinical types of diabetic. Plasma collected from diabetics presenting with weight loss and ketonuria before insulin treatment possessed insulin activity comparable to that of plasma from normal people. These observations confirm those of Groen and his co-workers with the same technique (Groen, J., Kamminga, C. E., Willebrands, A. F., and Blickman, J. R. (1952), *J. clin. Invest.*, **31**, 97). I have also studied the insulin activity of plasma from two pregnant diabetics. There the levels are higher than normal and comparable to those seen in acromegaly. I have yet to study the insulin activity of plasma from non-diabetic pregnancies, so that I cannot say whether the enhanced plasma insulin activity seen in diabetic pregnancy is an abnormal feature.

G. Gori: Are these people injected with insulin, and if not, for how long?

Randle. The ordinary diabetics were not injected with insulin, they had never been given insulin at all.

Park: If you have a depancreatized animal and inject a known dose of insulin and then assay the plasma afterwards, how much of that insulin can you account for?

Randle: I have not done that.

Lawrence: We did a few untreated and found in the ketosed low insulin,

as was the sample collected from a non-acromegalic diabetic who acted as one of the controls. I have studied, to date, about twenty-five acromegalics, and some eighteen of these show definitely enhanced plasma insulin activity. The vast majority of these cases of acromegaly are non-diabetic.

Fod: Would you comment on the parallelism between the effect of growth hormone which you have pointed out and its diabetogenicity? Does growth hormone cause primarily hyperglycaemia which is then followed by an increased insulin production, or does it stimulate the pancreas to produce more insulin followed by "exhaustion" diabetes?

Randle: I think it suggests that growth hormone stimulates insulin secretion by causing hyperglycaemia but it does not prove this. I should emphasize that my own experiments do not exclude the possibility that growth hormone stimulates the secretion of insulin by the pancreatic islets of the rat. They merely show that there is no detectable

[illegible]

studies?

Randle: Anderson and Long added 0·08–1 mg. of growth hormone to 10 cc. of perfusing blood. In my experiments the last injection of growth hormone was never greater than 10 mg. to a 4-kg. cat.

[illegible]

different.

Randle: Yes, definitely.

Young: Do you feel safe, Dr. Randle, in deducing that growth hormone does stimulate secretion of insulin by the islets of Langerhans in the cat?

THE CONTROL OF THE SECRETORY ACTIVITY OF THE ISLETS OF LANGERHANS*

PIERO P. FOÀ

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The Secretion of Insulin

1. Nervous control

Claude Bernard suggested in 1849 that nervous centres located in the floor of the fourth ventricle regulate the liberation of glucose from the liver via the sympathetic nerves. The observations of Bernard were confirmed and extended by numerous investigators whose work, reviewed recently by Anderson and co-workers (1952), led Macleod (1934) to postulate the existence of a "diabetogenic centre". A great diversity of conditions can cause a temporary decrease in carbohydrate tolerance, glycosuria, and increased insulin resistance. Some of these conditions are lesions in the cerebellum, the brain stem or the hypothalamus, section of the brain stem (Anderson *et al.*, 1952), section of the hypothalamic pathways with adrenal demedullation (Beattie, 1954), or just head injury (Ruesch and Bowman, 1944), brain tumours (Paton and Petch, 1954), the outburst of emotions and sham rage (Gellhorn *et al.*, 1941; McCullagh, 1954), or hypothalamic lesions with changes in food consumption and obesity (Kennedy, 1952; Ranson *et al.*, 1938).

The common mechanism of action of these conditions, if one exists, is not understood, and Macleod's centre may turn out to be not an anatomic entity, but a number of physiological mechanisms regulating the sympatho-adrenal system, epinephrine secretion, the rate of liver glycogenolysis and,

* Aided by grants from the United States Public Health Service and from Mr. Simon Solomon.

and as soon as we treated with insulin we got them back to what we
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insulin in a subsequent test. I think that specificity is of paramount importance when a bioassay is used to measure the level of a hormone in a complex fluid such as blood plasma, unless one can first extract the hormone from the plasma in reasonably pure form. We know very little about the state of hormones in plasma, but they do not necessarily exist in plasma in the form in which they have been extracted from the parent gland. The specificity of a bioassay, as applied to plasma, is not necessarily established, therefore, by studies with hormones in the form in which they are extracted from their parent gland.

Waugh: If you plot the log.dose response curve, is it a straight line for plasma?

Randle: I have only studied three concentrations of plasma with samples from normal fasted rats. There the log.dose response curve for

100 : 1 one realizes most of the anti-thrombin, but at an intermediate dilution there is a 70 per cent drop.

Young: Your figures for the insulin content of blood, based on the activity of normal plasma, are considerably higher than those found by others.

Randle: That is so. The values which I have obtained for the insulin activity of normal human plasma are of the order of 10 milliunits of insulin per ml., which is twenty to thirty times greater than values obtained for untreated plasma by methods of insulin assay based upon the blood sugar response of suitable test animals.

glycaemic by continuous glucose injection, whereas it is active in a depancreatized animal similarly treated (Jordan, 1947). Direct evidence of the effect of hyperglycaemia on insulin secretion was obtained by anastomosing the pancreatic vein of one dog with a systemic vein of another (Zunz and LaBarre, 1927*b*; Foà *et al.*, 1949), by grafting a second pancreas in the neck of a dog and injecting glucose into the pancreatic artery (Houssay and Deulofeu, 1939), by injecting glucose into the artery of a pancreas left *in situ* (Brown *et al.*, 1952), and by perfusing an isolated pancreas with glucose-containing fluid (Anderson and Long, 1947*a*). The chronic intraperitoneal administration of glucose also appears to stimulate the islets of Langerhans in normal rats (Kinash and Haist, 1954*a* and *b*) and to cause hypertrophy and hyperplasia of the pancreatic remnants in rats with subtotal pancreatectomy (Houssay *et al.*, 1947), while high carbohydrate diets appear to accelerate the restoration of granules in β-cells inhibited by the administration of insulin or by starvation (Nerenberg, 1953*a* and *b*). Although, under special conditions, the prolonged intravenous administration of glucose may cause pancreatic necrosis (Jacobs and Colwell, 1936), in most cases, the stimulation of the islets of Langerhans is followed only by degeneration and degranulation of the β-cells and, sometimes, by hyperglycaemia and glycosuria (Dohan and Lukens, 1948; Wissler *et al.*, 1949; Butturini and Baronchelli, 1953; Haist, 1953). On the other hand, caloric restriction and restriction of carbohydrate intake reduce islet activity (Haist, 1953), and the avoidance of hyperglycaemia by means of insulin or phlorhizin protects the islets from the effects of excessive carbohydrate. This work, which was reviewed by Lukens (1944) and by Ingle (1948), suggests that overstimulation may cause failure of the insulin-secreting mechanism, especially when the pancreatic reserve is decreased.

(*b*) *Effect of Other Sugars.* Little is known of the effect on insulin secretion of carbohydrates other than glucose. Nerenberg (1953*a* and *b*) obtained evidence of β-cell stimulation by galactose, fructose, sucrose, and lactose given orally,

perhaps, food consumption and the secretion of ACTH (Hume and Nelson, 1955), insulin and other hormones. The initial stimulus for the activation of the sympatho-adrenal system appears to be hypoglycaemia which causes secretion of epinephrine, as indicated by depletion of the adrenal medulla (Hokfelt, 1953), increase in the epinephrine content of the adrenal blood (Duner, 1954) and in urinary epinephrine excretion (Euler and Luft, 1952). In addition the central nervous system may influence the endocrine activity of the pancreas by means of vagal impulses, the actions of which are still uncertain (Phillips, 1933; Gregory *et al.*, 1948). Thus, according to Zunz and LaBarre (1927*a*, 1928), hyperglycaemia acts on the vagal centres causing stimulation of the pancreas and an increase in insulin secretion, while according to others (Gayet and Guillaumie, 1933; Foglia and Fernandez, 1935), hyperglycaemia stimulates insulin secretion independently of extrinsic pancreatic innervation. The conclusion of Anderson and co-workers (1952) that a central nervous system mechanism for the homeostatic control of blood sugar level may exist still appears valid. Many details of this mechanism remain to be clarified, although it appears probable that its function in the adjustment of blood sugar level is an accessory and not a necessary one. At the present time there is no indication that the nervous system exerts any lasting effects on the endocrine activity of the pancreas and that it is involved in the pathogenesis of human or experimental diabetes.

2. Metabolic control

(*a*) *Effect of Glucose.* The slow intravenous injection of 10 per cent glucose into normal persons induces a rapid removal of glucose from the blood, so that during the latter part of the injection the blood sugar level, instead of continuing to increase, actually decreases (Thalhimer *et al.*, 1926). This phenomenon, like the hypoglycaemic phase of the glucose tolerance curve, suggests that hyperglycaemia stimulates insulin secretion (see references in Foà, 1949). For this reason, insulin has very little effect in normal dogs rendered hypo-

plasma insulin regulates insulin secretion, insulin destruction (Mirsky, 1953; Elgee and Williams, 1955*a* and *b*) may also contribute to the regulation of the activity of the pancreatic islets.

(*b*) *Effect of Glucagon.* The injection of glucagon may stimulate insulin secretion. This statement is based on the following evidence: (1) the hyperglycaemic effect of glucagon is smaller in the presence of the pancreas than in its absence (Foà *et al.*, 1952*a*); (2) the injection of glucagon causes the appearance of a *blood-sugar lowering material (insulin) in the* blood of the pancreatic vein (Foà *et al.*, 1952*b*); and (3) rats with the obese-hyperglycaemic syndrome appear to be suffering from excessive glucagon secretion (Mayer *et al.*, 1953) and have higher than normal quantities of insulin in their pancreas (Wrenshall *et al.*, 1955). It is impossible to say whether these effects are due to a direct action of glucagon on the pancreas or whether they are the result of the accompanying hyperglycaemia. The fact that chronic treatment with glucagon does not alter the appearance of the islets of Langerhans (Galansino *et al.*, 1955) militates against the first alternative.

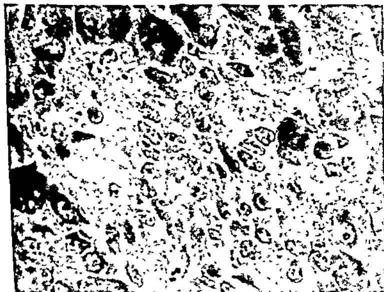
(*c*) *Effect of Growth Hormone (GH).* The effect of GH on the islets of Langerhans has been extensively reviewed (Young, 1953; Houssay *et al.*, 1953; Campbell, 1955). GH appears to inhibit the secretion of insulin by the isolated pancreas in response to glucose (Anderson and Long, 1947*b*). Prolonged treatment with GH may act on the pancreas directly or it may act indirectly by increasing the demand for insulin. In some animals (rats, puppies), the pancreas responds to this stimulus with signs of increased activity, such as hypertrophy of the islets (Haist, 1953; Kinash *et al.*, 1953), increased plasma insulin (Randle, 1955), increase in glycogen deposits (Illingworth and Russell, 1951) and hypoglycaemia (Milman and Russell, 1950). In other animals (adult dogs, or animals with decreased pancreatic reserve) excessive stimulation by GH causes degranulation of the β-cells, decrease in the insulin content of the pancreas, and severe diabetes (Campbell *et al.*, 1953; Campbell, 1955).

and by galactose and fructose given parenterally. However, since the experiments were performed in intact animals, it is possible that these sugars may have been converted to glucose by the liver. The oral administration of galactose to infants with galactosemia, whose liver is unable to convert galactose to glucose, is followed by a sharp decrease in the concentration of blood glucose (Bruck and Rapoport, 1945; Goldbloom and Brickman, 1946), *suggesting an increase in insulin secretion.* Sodium mesoxalate injected into the pancreatic artery causes hypoglycaemia, suggesting that this compound also may stimulate insulin secretion (Kobayashi and Ohashi, 1955).

3. Endocrine control

(*a*) *Effect of Insulin.* The administration of a hormone may inhibit the activity of the gland which secretes it. Thus, ovulation may be inhibited by oestrogen, hypothyroidism may follow the administration of thyroxine, and glucose tolerance may be temporarily decreased by the administration of insulin (Clark *et al.*, 1935; Looney and Cameron, 1937). Zunz and LaBarre (1927*c*) observed that the pancreatic blood of a donor dog in insulin hypoglycaemia no longer lowers the blood sugar of a depancreatized recipient and suggested that insulin hypoglycaemia inhibits insulin secretion. This hypothesis was confirmed in a more convincing fashion by the observations that as many as three additional pancreases can be grafted into an animal without causing hypoglycaemia (Houssay *et al.*, 1955), and that insulin administration is followed by: (1) decrease in the insulin content of the pancreas (Best and Haist, 1941); (2) inhibition of islet growth (Evans and Haist, 1951); and (3) degranulation of the β-cells with temporary diabetes (Barron, 1948; Nerenberg, 1953*a*; Bell, 1953). In this case, contrary to the situation existing after excessive insulin demand, degranulation does not indicate overstimulation and exhaustion, but functional inactivity. Under these circumstances, the administration of exogenous insulin may allow the restoration of the β-cell granules (Copp and Barclay, 1923; Hausberger and Ramsay, 1952). If

FIG. 2 Islet of Langerhans of a normal, untreated dog; Gomori stain, ×530.



et of Langerhans of a normal dog one hour after the injection of prolactin (20 I U /kg I V). Gomori stain, ×530.

facing page 50

(*d*) *Effect of ACTH and the Adrenocortical Hormones.* The mechanism of the diabetogenic action of ACTH and of the adrenocortical hormones has been reviewed recently (Long, 1953; DeBodo and Sinkoff, 1953*a*) and is not fully understood. [illegible] Krahl, 1953), and increase gluconeogenesis (Long, 1953). They, or the hyperglycaemia which they cause, may stimulate the islets of Langerhans (Hausberger, 1953; Abrams *et al.*, 1953; Bates, 1953; Haist, 1953; Kinash and Haist, 1954*a* and *b*; Hausberger and Ramsay, 1955), leading sometimes to hypertrophy of the islets (Houssay *et al.*, 1954) with increased insulin content (Wrenshall and Ritchie, 1952), and sometimes to degranulation, degeneration and disappearance of the β-cells. This diabetogenic effect has been related to a reduction in the supply of thiol groups necessary for the activity of many enzymes concerned with glycolysis and phosphorylation (Anderson *et al.*, 1951; Conn, 1953; Bacila and Guzman-Barron, 1954), and for the protection of the β-cells against the damaging effects of alloxan and alloxan-like material (Martinez, 1951; Conn, 1953; McCullagh and Alivisatos, 1954).

(*e*) *Effect of Prolactin.* Early contradictory studies on the effect of anterior pituitary extracts containing prolactin have been reviewed elsewhere (Foà, Galansino and Costa, 1955; Foà [illegible] and hypophysectomized-adrenalectomized dogs. On the other hand, prolactin appears to stimulate the growth of the islets of Langerhans in the intact (Mount, 1951) and in the hypophysectomized animal (Haist, 1953). This stimulation may lead to degeneration of the insulin-secreting cells and to diabetes (Houssay *et al.*, 1954).

The influence of prolactin on blood sugar and on the islets of Langerhans of normal and depancreatized dogs was investigated in our laboratory (Foà, Galansino and Costa, 1955;

Foà *et al.* 1955). The first intravenous injection of prolactin (Armour lot R-491154; 20 I.U./Kg.)* into normal dogs causes a prompt and significant decrease in blood sugar (Fig. 1), associated with a profound degranulation of the β-cells (Figs. 2 and 3). Pancreatic femoral cross-circulation experiments between a donor *Dog D* and a recipient *Dog R* showed that hypoglycaemia caused by the first injection of prolactin

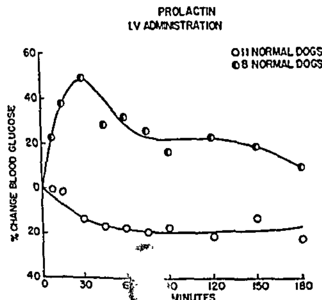


FIG. 1. Effect of prola[illegible] ...ood sugar of normal dogs. o: first injection [illegible] ...nd fourth injections.

...to *Dog D* is accompanied b[illegible] ...r hypoglycaemia in *Dog R* ...4), indicating that [illegible] ...nulation of the β-cells is ...anied by lit[illegible] ...n. Subsequent prolactin ... a rise in b[illegible] (Fig. 1), associated with ... of the β [illegible] ...g. 5). Cross-circulation ... [illegible] ...logs and untreated re... ...this [illegible] ...a is not accompanied

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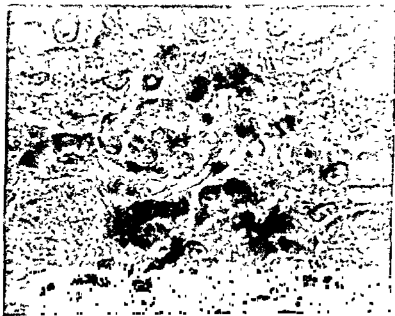


Fig 5 Islet of Langerhans of a normal dog after 5 injections of prolactin (20 I U /kg I V) at 2–4-day intervals, Gomori stain, ×530

Foà *et al.* 1955). The first intravenous injection of prolactin (Armour lot R-491154; 20 I.U./Kg.)* into normal dogs causes a prompt and significant decrease in blood sugar (Fig. 1), associated with a profound degranulation of the β-cells (Figs. 2 and 3). Pancreatic femoral cross-circulation experiments between a donor *Dog D* and a recipient *Dog R* showed that hypoglycaemia caused by the first injection of prolactin

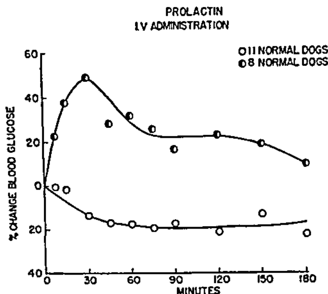


FIG. 1. Effect of prolactin on blood sugar of normal dogs
o: first injection; ●: third and fourth injections

in *Dog D* is accompanied by a similar hypoglycaemia in *Dog R* (Fig. 4), indicating that the degranulation of the β-cells is accompanied by liberation of insulin. Subsequent prolactin injections cause a rise in blood sugar (Fig. 1), associated with partial restoration of the β-granules (Fig. 5). Cross-circulation experiments between pretreated donor dogs and untreated recipients indicate that this hyperglycaemia is not accompanied

* Gift of Drs. I. Bunding and S. R Steelman of the Armour Research Laboratory.

by the liberation of hyperglycaemic material (glucagon) in the blood of the pancreato-duodenal vein (Fig. 6), nor

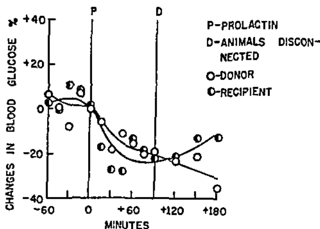


FIG 4 Effect of prolactin on blood sugar of untreated dogs; pancreatic-femoral anastomosis, 5 experiments.

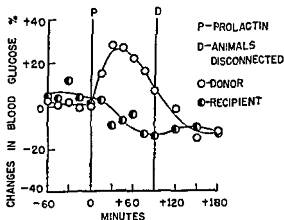


FIG 6. Effect of prolactin on blood sugar of dogs. Donor dogs pretreated with prolactin, recipient dogs untreated Pancreatic-femoral anastomosis, 5 experiments.

by changes in appearance of the α-cells or in the α : β-cell ratio. DeBodo and Sinkoff (1953*a* and *b*) did not observe a

lowering of the blood sugar following the first intravenous injection of prolactin into hypophysectomized dogs. This may be due to the fact that the islets of hypophysectomized dogs are already partially atrophied (Kinash *et al.*, 1953) and have a smaller insulin content. In agreement with this hypothesis, prolactin does not cause hypoglycaemia in normal dogs after the β-cells have been partially degranulated by repeated

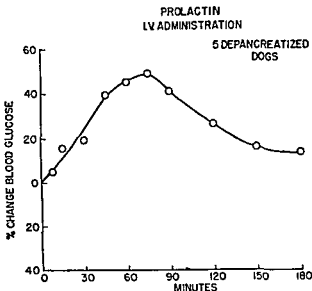


FIG. 7 Effect of prolactin on blood sugar of untreated depancreatized dogs.

injections or when the pancreas has been removed; on the contrary, in the depancreatized dog a marked and significant rise in blood sugar level occurs after the first injection of prolactin (Fig. 7). This hyperglycaemic effect is unexplained at the present time; it cannot be due to the liberation of hyperglycaemic hormones of pituitary, adrenal or pancreatic origin, since it is observed also in the absence of these glands. The possibility that prolactin may inhibit glucose uptake by the tissues has not been investigated, while the possibility

that the results may have been due to the technique employed or to ACTH, GH or posterior pituitary impurities present in the prolactin preparations was ruled out by control experiments.

(*f*) *Effect of the Sex Hormones.* The effect of the gonads on experimental diabetes was studied recently by Houssay and co-workers (1954). According to these investigators, the incidence of diabetes following subtotal pancreatectomy is much greater in male than in female rats, and castration increases the incidence in females and decreases it in males. The administration of oestrogens to force-fed alloxan diabetic rats at first causes an exacerbation of diabetes (Ingle *et al.*, 1947), but, if treatment is prolonged, the incidence and severity of diabetes decrease, the remaining islets become hypertrophic and as many as 47 per cent of the rats are cured. If insulin is also given, the number of animals cured rises to 69 per cent (Rodriguez, 1954). The antidiabetic effect of oestrogens can be demonstrated also in the capon (Sturkie, 1955) and in acromegalic patients in whom oestrogens correct the abnormal glucose tolerance (McCullagh *et al.*, 1955). This effect of oestrogens may be due to stimulation of islet growth (Kerr *et al.*, 1952; Haist, 1953; Rodriguez, 1954) or to inhibition of GH production by the anterior pituitary (McCullagh *et al.*, 1955). Progesterone and some of its derivatives aggravate experimental diabetes (Ingle *et al.*, 1953*a* and *b*; Houssay *et al.*, 1954*a* and *b*), although progesterone may stimulate islet growth (Haist, 1953). Androgens also increase the incidence and accelerate the onset of diabetes in rats with subtotal pancreatectomy (Houssay *et al.*, 1954), and perhaps for this reason the anabolic effect of testosterone is smaller in diabetic than in normal dogs (Sirek and Best, 1953).

(*g*) *Effect of Thyroid Hormone.* The administration of thyroid substance causes an increase in weight of the islets of [illegible] (Haist [illegible]

[illegible]

b). Normal pancreas seems to tolerate this stimulation quite

well; however, if the pancreatic reserve is reduced by partial pancreatectomy or by the injection of anterior pituitary hormones the administration of thyroid hormone causes lesions of the β-cells. These lesions are temporary at first, but may become permanent if treatment is continued (Houssay, 1945).

In conclusion it can be said that, although the manner in which nervous, metabolic and hormonal factors influence the activity of the insulin-secreting cells is not fully understood, all these factors may have hyperglycaemia and increased insulin demand as the common denominator. Hyperglycaemia *and insulin demand are physiological stimuli for the* β-cells, and if they are not excessive and the pancreatic reserve is good, they cause hypertrophy, hyperplasia and other signs of β-cell overactivity. On the other hand, if these stimuli become excessive or the pancreatic reserve is low, degranulation of the β-cells and reduced insulin content of the pancreas and diabetes may result (Bell, 1953; Wrenshall *et al.*, 1955; Hartroft and Wrenshall, 1955).

The Secretion of Glucagon

1. Nervous control

No studies of the glucagon content of the pancreas or of the pancreatic blood after nervous stimulation or interruption of nervous impulses to the pancreas are available. Sergeyeva (1943) noted that vagotomy increases and sympathectomy decreases the α : β-cell ratio in the cat. However, this phenomenon could be due to a change in the number of β- rather than α-cells.

2. Metabolic control

Cross-circulation experiments (Foà, 1954; Foà and Galansino, 1954) have shown that insulin hypoglycaemia is followed by the appearance of a hyperglycaemic material (glucagon) in the pancreatic, but not in the mesenteric blood, and

suggested that hypoglycaemia may be a physiological stimulus for the secretion of glucagon.

3. Endocrine control

(*a*) *Effect of Insulin.* In agreement with the hypothesis that insulin (or hypoglycaemia) may stimulate glucagon production, MacGrath and Snedecor (1953) found an increase in the glucagon content of the pancreas of insulin-treated animals. Prolonged insulin treatment is followed by an increase in the number of α-cells in the islets of Langerhans (Hagemann, 1953); however, since insulin may cause a decrease in the number of β-cells and since not all investigators agree that the α-cells are the site of origin of glucagon, the significance of this observation cannot be evaluated at the present time.

(*b*) *Effect of Glucagon.* Bensley and Woerner (1938) reported that the intravenous injection of an extract of α-cells causes histological signs of α-cell inactivity in the guinea pig. However, the specificity of the extraction and histological methods used is open to question and the results are difficult to interpret. Involution of the α-cells after administration of glucagon was observed by Kracht (1954), but not by Galansino and co-workers (1955), who, after daily injections of glucagon for as long as 66 days, could find no significant changes in the appearance of the α-cells or in the value of the α : β-cell ratio in rats. Should future work indicate that the concentration of glucagon in the blood contributes to the regulation of its own secretion, the mechanisms for glucagon destruction (Goldner *et al.*, 1954; Van Arsdel *et al.*, 1954) would acquire greater significance.

(*c*) *Effect of Growth Hormone (GH).* Hypophysectomy causes involution of the α-cells (Ferner and Tonutti, 1953). GH corrects this involution (Kracht, 1953) and causes a temporary increase in the α : β-cell ratio followed, after six weeks of treatment, by degranulation of the α-cells (Volk and Goldner, 1955). GH appears to cause an increase in size of the α-cells in normal rats (Abrams *et al.*, 1953) and an increase in

the number of α-cells undergoing mitosis in normal rats (Cavallero, 1953*a*), in mice with pituitary dwarfism (Cavallero, 1953*b*) and in rats treated with colchicine (Cavallero and Mosca, 1953), suggesting that GH may have a trophic effect on the α-cells of the islets of Langerhans. In agreement with this hypothesis, Bornstein and co-workers (1951) found that the pancreatic blood of cats treated with GH causes hyperglycaemia in alloxan diabetic hypophysectomized-adrenalectomized rats, and Foà and co-workers (1953), by means of cross-circulation experiments, found that single intravenous injections of growth hormone cause the appearance of a hyperglycaemic material, presumably glucagon, in the pancreato-duodenal blood of dogs. Glucagon secretion may explain in part the hyperglycaemic effects of intravenous injections of growth hormone (Carballeira *et al.*, 1952; Kinsell *et al.*, 1953; Foà *et al.*, 1953; Raben, 1955), especially since this hyperglycaemia appears to be associated with an increased glucose output by the liver (Kibler *et al.*, 1952; Engel, 1955), which is a characteristic glucagon effect. In rats with the obese-hyperglycaemic syndrome, hypersecretion of glucagon is believed to occur under the influence of growth hormone *(Mayer et al., 1953). On the other hand, Bennett (1955) could* find no evidence of glucagon secretion following the injection of GH. This author studied the problem by grafting a normal pancreas in the neck of a depancreatized dog and observing the rate at which the blood sugar of the dog returned to normal.

Bennett's experiment would have been more convincing if the pancreas had been grafted into the neck of a normal rather than a diabetic dog and had been perfused with normal rather than hyperglycaemic blood, since hyperglycaemia tends to promote the secretion of insulin and mask the action of glucagon.

The relationship of GH to glucagon, like other problems of glucagon physiology discussed in this Colloquium, requires further study before the hormonal nature of this material and its importance in blood sugar haemostasis can be fully understood.

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Fod: Am I correct in saying that you, as well as Dr. Houssay, have

hormone.

treated animals the pancreas was secreting insulin at a very much greater rate than normal, and therefore the intravenously injected insulin was less effective than normal since it constituted only a small proportion of

87, 15*P*, 1936, *Lancet*, 1, 297).

What were your time intervals when you gave repeated prolactin injections to normal dogs and got hypoglycaemia?

Fod: One or two days.

Randle: Have you given prolactin to the acutely depancreatized dog to see whether it would produce a fall in blood sugar under those conditions?

Fod: I do not know what you mean by acutely depancreatized. These

DISCUSSION

Randle: Have you managed to induce even temporary diabetes in intact dogs with prolactin?

... as many as four times,
... ut noticing any
... ucose-tolerance
... s.
... STH? I think
... ed very similar

at intervals of ...
response to two units of intravenous insulin, the insulin being ...
intravenously about 3 hours after the second injection of prolactin
... blood sugar level had not risen under those conditions and yet
... in that effect

Marks found that the prolactin ...
... of insulin in the eviscerated cat (1936, *J. Physiol.*, 87, 1...
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different preparations greatly ... ine
... prolactin itself. Later, when we invest

THE EFFECTS OF HYPOPHYSECTOMY AND OF PROLONGED GROWTH HORMONE ADMINISTRATION ON THE PANCREATIC α-CELLS OF THE RAT

MARTIN G. GOLDNER AND BRUNO W. VOLK

Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn, New York

THE diabetogenic effect of crude anterior pituitary extracts and of purified pituitary growth hormone has led repeatedly to studies of the direct action of the hormones of the adenohypophysis on the pancreatic islets. So far, however, no definitive evidence of the existence of a pituitary pancreatotropic hormone has been presented (Best, Haist and Wrenshall, 1955).

In particular, there has been considerable discrepancy of experimental data and of interpretations as to the existence of a functional relationship between the pituitary and the pancreatic α-cells. While some authors postulate an α-cytotropic pituitary action based on direct or indirect experimental evidence, other investigators have been unable to come to similar conclusions (Volk, Lazarus and Goldner, 1954). These contradictory observations may at least in part be due to one or all of three factors: (1) species variation in response to growth hormone; (2) differences in the activity of various growth hormones, and (3) technical difficulties in the differential stains of the islet cells. The Gros-Schultze stain, for instance, which is widely used for the silver impregnation of the α-cells, is known to produce different results in the hands of various investigators. The capriciousness of this method causes such variability of the demonstrable silver cells that different counts may be obtained even in the same tissue block, depending on minor changes in the timing of the silver

...ments as early as 6 hours.
...vel to the stimula-
...seems to be almost
...hat the stimulation
...ontent of the fluid
...relationship to the

phatase?

de Duve: We have not looked for it in that tissue.

G. Cori: If it does not, then you would not get any sugar from
... time. We

Mosca: We ... at some capillaries of the islets cross fields of β-cells before reaching α cells and ... Other capillaries can cross α-cells and collect the α-cell ... that the blood

...Randle
...l in
...ast-
...lear
...and
this fall was completely ... or two large doses of growth hormone This phenomenon may be associated with residual insulin in the tissues.

of the animals were fixed in Bouin's solution. Serial sections cut at 7 microns were stained alternately by Gomori's chrome alum haematoxylin and phloxin technique (Gomori, 1951) and by a modification of Davenport's silver impregnation. This latter modification, developed in our laboratory, does not depend on subjective factors as the Gros-Schultze technique does and gives reproducible results. It permits the use of Bouin-fixed material and thus the alternate staining of serial sections by this and by the Gomori technique. The procedure, described in detail elsewhere (Volk, Goldner and Frank-Crowley, 1955), is as follows:

1. Deparaffinize by running sections through xylene (2 jars, each 15 minutes) followed by the alcohols and by distilled water.
2. Refix in Bouin's fluid for 18–24 hours.
3. Wash under the tap for 1·5 hours.
4. Oxidize sections in the following mixture for one minute: potassium permanganate 0 3 g., distilled water 100 ml., sulphuric acid 0·3 ph.*
5. Decolourize with 3% solution of sodium bisulphate. Wash under tap for 30 minutes.
6. Run section back to 95% alcohol and immerse in Davenport's silver solution consisting of 10 g. of silver nitrate dissolved in 10 ml of distilled water, 90 ml. of 95% alcohol and 0 5 ml. of N-nitric acid. Immerse sections at room temperature for 40–48

10. Dehydrate with absolute alcohol, and xylol and mount in balsam. Results: silver cells appear black, β-cells—pale brown, the exocrine portion—dark brown.

Results

Group 1. In the pancreatic islets of the normal rat stained with the Gomori technique, the α-cells are located at the periphery while the β-cells occupy the rest of the islet. In corresponding sections stained with the Davenport modification, the silver-impregnated cells have a peripheral distribution within the islets similar to the α-cells visualized by the

* The oxidation process is that used for Gomori's chrome alum haematoxylin and phloxin method

impregnation (Creutzfeldt, 1953). The histological observations, therefore, cannot reliably be compared with the results obtained by the use of other techniques for the demonstration of the pancreatic islet cells.

Because of the discrepancies in morphological and physiological findings, and because of the considerable importance of the question of a possible functional relationship between the anterior pituitary and the pancreatic α-cells, we undertook to study the $\alpha : \beta$-cell relationship in pancreatic islets of normal and hypophysectomized rats and in a series of such animals subjected to prolonged treatment with growth hormone.

Material and Methods

For this study adult white rats of either sex of the Sprague-Dawley and Wistar strains were used, weighing 200–250 g. The animals were divided into three groups. Group 1 consisted of 20 hypophysectomized rats which were allowed to live for 4·5 months after operation. Ten normal animals served as controls. Group 2 consisted of 4 normal and 4 hypophysectomized rats which received daily subcutaneous injections of growth hormone for periods up to 3 weeks. Group 3 consisted of 7 hypophysectomized and 2 normal rats receiving growth hormone as Group 2 but being treated daily for periods up to 6 and 10 weeks.

The growth hormone used was Somar Growth Hormone.* The diabetogenic action of this preparation had been confirmed in previous work on dogs by Volk, Lazarus and Co Tui (1952). The daily dose was 1 mg./200 g. body weight. The animals were weighed at regular intervals and blood sugar determinations were done at the same time. Individual animals of the growth-hormone treated groups were sacrificed at weekly intervals and the remaining animals at the end of the 3-week or 10-week periods. Completeness of the hypophysectomy was checked in each operated animal.

The pancreatic glands removed immediately after sacrifice

* Lot M-308, kindly supplied by Armour and Company through the courtesy of Dr. Irby Bunding.

of 26 per cent of all islet cells was identified as α-cells with a range from 23–31 per cent. The overall average of the corresponding silver-impregnated cells was only 7 per cent, with an individual range fluctuating between 5 and 8 per cent (Table I).

It might be stated that the hypophysectomized animals showed the same minor fluctuations in weight as the control animals and that the blood sugar values were well within the physiological range throughout the 4–5-month period.

Group 2. The islets of growth-hormone treated normal and hypophysectomized rats showed no changes from the controls for the first two weeks. At the 3-week period, however, an increased cellularity of the islets with a relative increase of the α-cells could be observed in both types of animals. In the hypophysectomized rats 36 per cent and 42 per cent respectively of the pancreatic islet cells were α-cells, in the normal animals 33 per cent and 34 per cent respectively. These values are beyond the range found in the larger groups of untreated normal and hypophysectomized rats, as shown in Table II.

Fig. 2 shows the pancreatic islets of a hypophysectomized rat treated with growth hormone for 3 weeks, and demonstrates that the islets are not only larger than normal but contain a relatively greater number of α-cells. The comparison of serial sections stained by Gomori stain and by silver impregnation also shows that not all α-cells visualized by the phloxin stain can be impregnated with silver.

Group 3. With continued daily administration of growth hormone for 6 weeks and longer, neither the normal nor the hypophysectomized animals showed any significant change in weight or in blood sugar homeostasis, but new and significant changes became noticeable in the islets. There developed a marked decrease in the number of α-cells, both absolute and relative. Consequently the α : β-cell ratio declined (Table III). Although the β-cells did not appear to be decreased in their number, they showed frequently considerable degranulation of the cytoplasm. Degranulation could be observed also in some of the remaining α-cells. This is demonstrated in

Gomori stain. However, not all α-cells can be impregnated with silver salts. This observation, which was made in all our specimens, is true also for the Gros-Schultze technique and raises the question whether the Gomori technique identifies two different cell types of which only one type can be silver impregnated. *From the serial slides it is evident that the cells*

Table I

PERCENTAGE OF ALPHA CELLS IN PANCREATIC ISLETS OF NORMAL AND HYPOPHYSECTOMIZED RATS, AS VISUALIZED IN GOMORI-STAINED AND SILVER IMPREGNATED SECTIONS.

STAIN	NORMAL		
	No of rats	Mean	Range
Gomori Stain	10	26%	23%-31%
Silver Impregnation		7%	5%-8%

STAIN	HYPOPHYSECTOMIZED*		
	No of rats	Mean	Range
Gomori Stain	20	27%	24%-31%
Silver Impregnation		7%	5%-8%

*Sacrificed 4 ½ months after hypophysectomy

identified by the modified Davenport technique belong to the group of Gomori's α-cells (Fig. 1*a*, *b*).

A comparison of the α : β-cell ratio of the pancreas of hypophysectomized rats surviving the operation for 4–5 months and of normal control animals did not show any appreciable difference by either the Gomori or the modified Davenport technique. In Gomori-stained sections an average

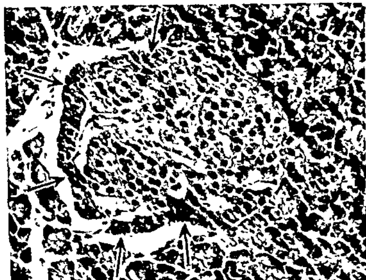




FIG 1 Pancreatic islet of a normal rat stained with (a) Gomori's chrome alum haematoxylin and phloxin method, and (b) the modified Davenport silver impregnation technique
Arrows indicate α-cells × 315

Table II

PERCENTAGE OF ALPHA CELLS IN PANCREATIC ISLETS OF NORMAL AND HYPOPHYSECTOMIZED RATS AFTER DAILY ADMINISTRATION OF GH FOR VARIOUS PERIODS OF TIME, AS VISUALIZED IN GOMORI-STAINED AND SILVER IMPREGNATED SECTIONS

STAIN	No. of rats	NORMAL, GH-TREATED		
		Sacrificed after		
		1 wk.	2 wks.	3 wks
Gomori Stain	4	28%	27%	33%, 34%
Silver Impregnation		7%	8%	9%, 10%

STAIN	No of rats	HYPOPHYSECTOMIZED GH-TREATED		
		Sacrificed after		
		1 wk.	2 wks.	3 wks.
Gomori Stain	4	28%	30%	36%, 42%
Silver Impregnation		8%	8%	10%, 12%

Table III

PERCENTAGE OF ALPHA CELLS IN PANCREATIC ISLETS OF NORMAL AND HYPOPHYSECTOMIZED RATS AFTER DAILY ADMINISTRATION OF GH FOR 6-10 WEEKS, AS VISUALIZED IN GOMORI-STAINED AND SILVER IMPREGNATED SECTIONS

STAIN	No of rats	NORMAL	HYPOPHYSECTOMIZED		
		Sacrificed after			
		6 wks	6 wks.	8 wks.	10 wks
Gomori Stain	9	17%, 20%	18%, 20%, 21%, 26%	20%, 24%	16%
Silver Impregnation	9	4%, 5%	2%, 3%, 3%, 5%	5%, 6%	3%

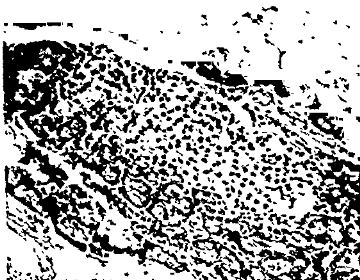




FIG. 3 Hyperplastic pancreatic islet of a hypophysectomized rat, treated with GH for 6 weeks (a) Gomori stain, the α-cells are decreased in number and are barely recognizable due to degranulation, (b) modified Davenport silver impregnation technique: there are but few silver impregnable cells present. × 210.



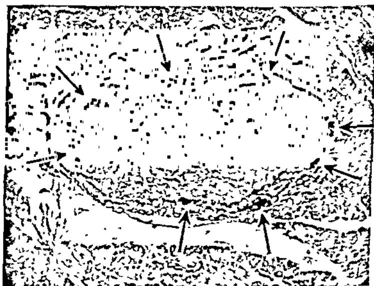


FIG. 2. Hyperplastic pancreatic islets of a hypophysectomized rat treated with GH for 3 weeks. (a) Gomori stain, (b) modified Davenport silver impregnation technique.

Arrows indicate α-cells. × 210.

Fig. 3, which shows corresponding sections, stained with the Gomori and the modified Davenport technique, of the pancreas of a rat treated with growth hormone for 6 weeks. In the Gomori stain there are hardly any α-cells noticeable, while the Davenport modification demonstrates a few peripherally located silver cells. The same can be seen on the Gomori stained section in Fig. 4. Here the islets seem to consist of β-cells only. Occasionally, the islets of this group of long-term treatment with growth hormone showed extensive necrosis in the presence of an intact exocrine portion. Both α- and β-cells in these islets have lost their staining characteristics. In view of this finding, it is noteworthy that none of the animals became diabetic. The functional reserve of the remaining islets was apparently sufficient to maintain blood sugar homeostasis.

Conclusions and comment

The observation that adult hypophysectomized rats remain alive for long periods of time (4–5 months) without appreciable changes in their blood sugar level and in the morphology of the pancreatic islets, seems to indicate that in the rat the pituitary is not essential for blood sugar homeostasis and for the endocrine function of the pancreas, at least as far as carbohydrate metabolism is concerned. We have not been able to confirm the cellular changes in the pancreas of hypophysectomized rats as seen by other investigators. Thus, Ferner and Tonutti (1953) observed a decrease in the number of α-cells without any changes in the β-cells, and Kracht (1953) found pyknosis of both cell types. Differences in staining techniques or in the developmental stage of the rats may be responsible for these disparate findings. While young rats after hypophysectomy show retarded general growth and retarded growth of the pancreas (Bryans *et al.*, 1952), the adult animals in our series showed no differences in their body weight. We did not check the weight of their pancreatic glands.

A word should be said here about the difference in the number of identifiable α-cells depending on the staining technique

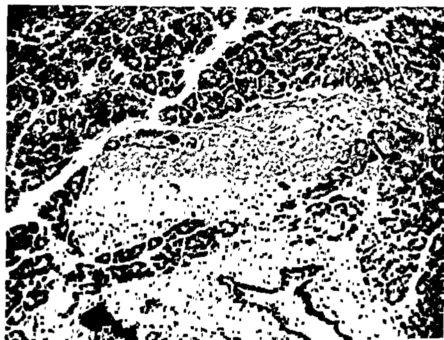


FIG 4 Pancreatic islet of a hypophysectomized rat, treated with *GH for 6 weeks There is marked degranulation and necrosis of the α- and β-cells present* Gomori stain × 120.

mass as described by Kinash and co-workers (1953) may be reflected in the increase in the individual islets during the hyperplastic phase in our animals. These findings raise the question of their physiological significance. Here four factors must be taken into consideration and should make us cautious in our interpretation: (1) The dosage of growth hormone used was unphysiologically large. (2) The same response was obtained from normal and hypophysectomized rats. (3) The response occurred only after rather prolonged treatment. (4) The morphological changes were not accompanied by any appreciable functional changes as far as the carbohydrate metabolism is concerned.

The large dosage of growth hormone may be responsible for the similar reaction of the islet cells of the normal and the hypophysectomized animals; that is to say, that whatever physiological hormone action was present in the normal rats was wiped out or overshadowed by the overwhelming dose of exogenous hormone. This, then, may suggest at the same time that a physiological dose of growth hormone would be without any effect on the islet cells and would correlate our findings in the untreated normal and hypophysectomized animals with those in the treated group.

The delayed morphological response of the islet cells to the stimulation of growth hormone seems to indicate that this response is not a primary but an indirect one and is mediated by some metabolic changes which secondarily affect the islets. For the absence of any disturbance of blood sugar homeostasis, two alternative explanations can be offered. On the one hand, the possibility exists that the functional capacity of the remaining non-affected islet cells was sufficient to maintain a normal blood sugar level. The β-cells of the rat are well known to have such a high functional reserve. On the other hand, consideration must be given to the possibility that the late morphological changes in the islets are secondary to metabolic effects of the growth hormone not related to carbohydrate metabolism.

Several investigators have been able to demonstrate a

employed. Our observation that only a small part of the α-cells identified with Gomori's technique can be impregnated with silver holds true not only for the Davenport technique and our modification. Even with the Gros-Schultze stain, *in spite of its unreliability, generally smaller α-cell counts are* obtained than with the Gomori technique (Grobety, 1947). Hultquist, Dahlen and Helander (1948) found with their silver stain 7 per cent α-cells in the islets of the rat. From these different staining characteristics it seems likely that we describe as α-cells a group of different cells which may also have different functions. This problem deserves further investigation.

Our findings *after growth hormone administration are not* easily reconciled with the observation on the untreated hypophysectomized rats. Here indeed we saw a significant effect of the pituitary hormone on the pancreatic islets. The effect was identical in the hypophysectomized and the normal rat. In both groups there occurred a biphasic morphological reaction, although no changes in the blood sugar were observed. This morphological reaction is characterized by (1) its late onset, occurring only after 8 weeks of daily treatment, and (2) a transitory phase of cellular hypertrophy in which the α-cells participate more than the β-cells and *resulting in an increase in the islet size, and* (3) after 6–10 weeks of daily treatment, a phase of cellular exhaustion with marked hypo[illegible]
α-cells and to [illegible]
in agreement [illegible]
variance with others. Thiemer (1953), for instance, saw no changes in the α : β-cell ratio after growth hormone administration to the hypophysectomized rat, while Kracht (1953) saw a recovery of the α-cells with shrinkage of the nuclei of the β-cells, and Cavallero and Mosca (1954), using the karyoclastic action of colchicine, observed a marked increase in mitotic figures in the α-cells. All these latter findings are in line with one or the other phase of the islet cell changes in our series. Also the increase in total weight of the islet cell

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DISCUSSION

Ferner: We too found that silver impregnation in rats is not sufficient for the indication of α-cells, but this is not so in man and in other animals. We have also studied α-cells after hypophysectomy. Three years ago we found that in the rat, for the greater part, there are no changes, in some of the rats we found atrophy and a numerical decrease of the α-cells. This is quite different in guinea pigs. In guinea pigs the α-cells have large oval nuclei, the β-cells have small round nuclei. In guinea pigs we always found atrophy and a numerical decrease of the α-cells, beginning 4–6 weeks after hypophysectomy (Ferner, H., and Tonutti, E. (1953), *Z. Zellforsch.*, 38, 267).

Best: In the older experiments carried out in rats by Dr. Haist and his

significant decrease in the insulin extractable from the pancreas of the dog after hypophysectomy.

Young: In 1941 Mervyn Griffiths, who had been working with me, published the statement (1941, *J. Physiol.*, 100, 104) that the insulin content of the pancreas of the 80-g. rat is significantly increased by hypophysectomy, but that the pancreatic insulin content of older animals is diminished.

Best: Do you remember whether this increase in insulin content had the same time relationships as the general growth of hypophysectomized young rats?

Young: Yes, that is so With our rats hypophysectomized animals

hyperglycaemic factor appearing in the blood of the pancreatic vein after growth hormone administration (Bornstein, Reid and Young, 1951; Foà, Weinstein and Smith, 1949) and have implied that this factor originates from the pancreatic α-cells. Likewise, the α-cells have been implicated in the disappearance of hyperglycaemia after hypophysectomy in mice of the hereditary hyperglycaemic and obese strain (Mayer, 1953). These functional changes are said to occur almost immediately after pituitary extirpation or growth hormone administration. They have not as yet been observed in the rat. Merely the lack of time relationship between them and the morphological alterations seen in our experiments would rule out any possible causal connection. Moreover, these experiments, as did our previous studies in which cobaltous chloride was used to destroy the α-cells (Volk *et al.*, 1954), fail to establish any relationship between the pancreatic α-cells and the hyperglycaemic glycogenolytic factor of the pancreas, glucagon. Further studies will have to elucidate the significance of the demonstrated morphological changes in the pancreatic islets of the rat induced by growth hormone.

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Schweiz

E., and

very confusing. For instance, the hypophysectomized guinea pig fails to respond to growth hormone. Marks and I found many years ago that although the guinea pig pancreas has β-cells with large granules which respond to changes in the blood sugar level, the amount of insulin extractable and assayed by normal methods is very much smaller than it is in the rat or other laboratory animals (1940, *Nature, Lond.*, 146, 31) We have never been quite satisfied with the reason for that.

de Duve: I would like to recall observations which have been made on birds. At least ten times as much glucagon can be extracted from the pancreas of birds as from the pancreas of mammals. As Prof. Best

Goldner: Does glucagon change the blood sugar in birds?

but the

There is

the physiological state but are abnormal.

Desaulles: Do you think there could be antibody formation after such a long time?

Goldner: I do not know.

Young: We now have a few minutes in which to consider further any

hypophysectomized animals with growth hormone does not change

... the growth hormone treatment does not

... mized rats even after growth hormone injection, provided the food intake is always at the same level as it is in hypophysectomized untreated controls.

Moreover, the ...

reache

tion.

Gold ...

diabetogenic action of growth hormone. We hoped thus to avoid indirect influences resulting from an effect of the growth hormone on the β-cells and their insulin content. We were therefore so much more surprised to find these late changes in α- and β-cells which could not be related to changes in the blood sugar level.

Fod: It seems to me that if destruction of the α-cells were accompanied by changes in blood sugar or glucose tolerance it would be very significant, but the fact that they are not does not necessarily mean that we are not dealing with glucagon. We tend to compare this situation to that which exists in respect to insulin, forgetting that while there is only one insulin, there are several blood-sugar raising hormones In the absence of glucagon, epinephrine or anterior pituitary may take over and therefore we may not see the deficiency of glucagon as readily as we see the deficiency of insulin.

Sutherland: Certainly it is as an intact animal and I presume that the liver slice is more sensitive too

glucagon in blood, we were using an assay system which could almost measure glucagon in blood. Perhaps an increased sensitivity of tenfold would allow measurement of glucagon in plasma.

Young: These species differences are interesting and no doubt they will yield something significant in the long run, but at present they are

FRACTIONATION OF INSULIN

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CRYSTALLINE samples of insulin now used in many laboratories possess a high biological activity and have been purified in several ways. They can be considered as pure as most other commonly known proteins, but this does not mean that they satisfy all the criteria of a pure chemical substance. In the past few years it has been recognized that none of the best samples gave a perfect purity test, if they were tested under appropriate conditions. Most of the physicochemical studies were first carried out in acid media, where insulin has the highest stability, and in such solutions the protein seems very satisfactorily homogeneous, as shown by solubility, electrophoresis or ultracentrifugation experiments.

The beautiful study of Harfenist and Craig (1952) using countercurrent distribution showed that all samples are composed of two main components in variable proportions and a few ill defined "impurities". On the contrary, Porter (1953) did not find indications of heterogeneity by the sensitive method of partition chromatography. Timasheff, Brown and Kirkwood (1953) have found evidence that insulin is electrophoretically heterogeneous, in particular at neutrality and between pH 3 and 4. We reported preliminary results of ultracentrifugation of insulin at pH 8 in chloride solution: the patterns showed the presence of at least two components in several cases (Fredericq, 1953*b*). The solubility curves between pH 4 and 5 were characteristic of a pure substance or of a quite heterogeneous one, according to the buffer used or to the treatment of the crystals (Lens, 1948; Fredericq and Neurath, 1950). Waugh and co-workers, (1953) found that insulin samples from different lots show

cobalt in alloxan diabetic animals and did not find a destruction of the α-cells (Holt, C. v., and Holt, L. v. (1954), *Naturf.*, 9b, 319). In our experience the metabolic changes observed in rabbits after cobalt chloride injection arise as a result of an enzyme intoxication in the liver and an alteration of the adrenals. There is some evidence in the literature that the morphological changes in the islets of Langerhans

G., and de Duve, C. (1952), *Arch. int. Physiol.*, 60, 128) can be explained as signs of exhaustion due to an increased requirement of glucagon The hyperplasia of the same cell type in rabbits after repeated cobalt chloride injections could be consequent on this requirement. As to IPTD, Loubatières has shown that this drug can only act in the presence of the pancreas, and our own results (p. 14) point to the destruction of the α-cells as a cause of the metabolic changes arising after IPTD injection.

Young. So far we have had no indication of what could be seen with the electron microscope in the pancreas of animals which had received these various treatments. I wonder whether something has been done in this direction. At the moment there seems to be a gap between visible microscopy, which we have been dealing with, and electron microscopy. Has anything been done with the electron microscope

of insulin?

Mosca: In the field of normal morphology there are the experiments of Hartroft (Hartroft, W. S. (1950), *J. clin. Endocrin.*, 10/7, 828) on electron microscopy. He showed a difference in the shape and size

bolic demand and hormonal function of the islets in these seasons (Mosca, L. (1955), *Monit. zool. ital*, 63/1, 1.)

von Holt: In connection with this, may I mention the work of Dr. Maske on the zinc content of the granules of the β-cells (Maske, H. (1953), *Z. Naturf.*, 8b, 96) He has some evidence that the granules of the β-cells are a depôt form of insulin attached to zinc.

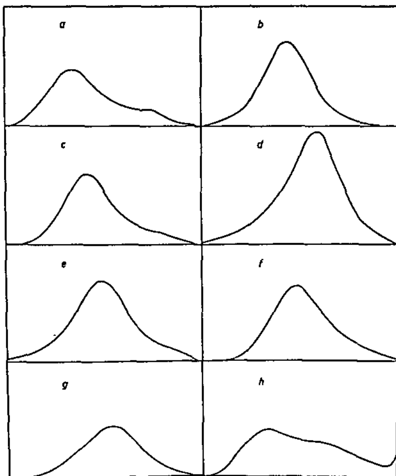


FIG. 1. Sedimentation patterns of insulin in 0 1 N-KCl (except *b*) at pH 8, after 2 hours of centrifugation at 60,000 r p m.

a Lilly T-2344
b. Lilly T-2344 in 0·1 N-phosphate buffer, pH 8.
c Lilly 200-1B-15.
d Lilly 200-1B-15, twice recrystallized
e. Boots
f Nordisk
g Boots fraction II.
h Boots fraction III

variations in fibril formation, crystallization and absorption spectra.

In general it can be said that heterogeneity is most evident from pH 4 to 8. In practice, between 4 and 6·5 the very low solubility precludes a physicochemical examination.

Few attempts at purification have been reported. The most successful was that of Harfenist and Craig (1952), who isolated by a great number of transfers in countercurrent distribution the so-called A and B components; they seem to differ by only one amide group (Harfenist, 1953). However, Harfenist and Craig (1952) have recognized the presence of at least a third active component. Timasheff, Brown and Kirkwood (1953), using electrophoresis convection of insulin and insulin-protamine complexes, have obtained fractions richer in some of the original components, but they were by no means pure. We have very few details on the work of Ellenbogen (1953), who announced the isolation of five different fractions, in liquid ammonia, with strikingly different molecular weights, solubilities and biological activities.

In fact, one can say that it has so far been impossible to prepare an insulin fraction behaving as a single component. Moreover, it is generally recognized that when an optimal point has been reached, repeated crystallizations of the samples increase the heterogeneity by some mysterious deteriorating effect.

In this paper we describe some more experimental studies on insulin heterogeneity and several attempts at fractionation based on solubility properties.

The heterogeneity of insulin

Fig. 1 shows a few sedimentation patterns of various insulin samples. They were photographed in a Spinco analytical ultracentrifuge on 1 per cent protein solutions in 0·1 N-KCl at pH 8, after about two hours of centrifugation at 60,000 r.p.m. At that pH the protein is still stable and has a relatively high molecular weight; dissociation occurs at a higher pH and is complete near pH 10 (Fredericq, 1953a).

are not the same for all the particles present in the solution, but that all of them nevertheless reach a complete dissociation with molecular units of the same size, at least in alkaline solutions.

Spectral modifications

Neutral or acid insulin solutions have an absorption spectrum with a maximum at 276 mμ and a minimum at 250 mμ (Fig. 2). Data from the literature reveal marked discrepancies for the molecular extinction near the minimum. We took as characteristic value the ratio of optical densities at 276 mμ and 250 mμ, which we call *R*. In Table I are shown *R* values *measured on* 0·1 *per cent insulin solutions in dilute phosphoric* acid, after 30 minutes of centrifugation at 14,000 r.p.m.

Table I

VALUE OF *R* FOR VARIOUS SAMPLES OF INSULIN
(*R* = ratio of optical densities at 276 mμ : 250 mμ)

Sample	*R*
Lilly T-2344	2 36
488098	2·25
535664	2·32
Boots	2 20
Nordisk	2 38
Novo	2·25

Dilution of these samples is always accompanied by a decrease in the value of *R*. No reversibility is observed when reprecipitating or lyophilizing the samples. In Table II are reported the *R* values for several concentrations of the Nordisk sample.

The value observed in the more concentrated solution corresponds to the molecular extinction that can be calculated from the amino acid content: the mixture of 6 moles of cystine, 8 of tyrosine and 6 of phenylalanine gives a value of *R* equal to 2·50 (calculated from the data of Beaven and Holiday, 1952).

The Lilly* samples show the presence of a minor heavier component with a sedimentation constant of about 5 Svedbergs, in proportions varying between 5 and 20 per cent. After two recrystallizations of lot 200-1B-15 the heavier component disappears but a spreading tendency on the left of the peak still shows some heterogeneity (Fig. 1 *a, c, d*).

In the Boots†, Novo‡, and Nordisk§ samples there is no appearance of a second peak; but in all of them there is a marked spreading tendency or a tail of the boundary (Fig. 1 *e, f*).

This heterogeneity can be semiquantitatively compared by determining an apparent diffusion constant of the protein, D_{app}, using the variation of the maximum height of the sedimentation boundaries with time. In the case of a monodisperse solute this apparent constant should coincide with the true diffusion constant under the same conditions of temperature and solvent. We found for Nordisk, $D_{app} = 16 \cdot 2 \times 10^{-7}$; for Boots, $15 \cdot 1 \times 10^{-7}$; and for Lilly recrystallized, $11 \cdot 6 \times 10^{-7}$. The true diffusion constant determined by Creeth (1953) on a Boots sample under similar conditions is $D = 8 \cdot 8 \times 10^{-7}$. Even the most heterogeneous sample (Lilly-T-2344), when examined under conditions of strong dissociation, i.e. at pH 2·7 or at *pH 10, gives patterns* characteristic of a single component. For a 0·25 per cent solution at pH 10, values of D and D_{app} were respectively 15 and $15 \cdot 3 \times 10^{-7}$.

Moreover, at pH 8 the shape of the boundary depends upon the salt medium: it looks more symmetrical in phosphate than in chloride (Fig. 1 *a, b*). Since in insulin solutions there could be molecules with different degrees of association, it is perhaps not surprising that at pH 8 polydispersity occurs. However, it is quite abnormal that this dispersity varies with the sample. It must be concluded that the dissociation constants at pH 8

* ... Dr. O. K. Behrens,

marked at higher pH. It has already been shown that the oxidation of tyrosine by permanganate or by tyrosinase has similar spectral effects (Haas, Sizer and Loofbourow, 1951). However, tyrosinase has very little action on insulin itself.

It is very likely that the decrease in the value of *R* is due to some oxidation of the tyrosyl residues by dissolved oxygen. The stirring of insulin solutions in the presence of air, particularly at pH 7, produces a strong decrease; *R* falls to 1·87 after one day and 1·4 after two days.

In the presence of antioxidants like diphenols in very small quantities, the decrease in the value of *R* is strongly inhibited (Table III).

Table III

VALUE OF *R* FOR AN 0·02 PER CENT BOOTS SAMPLE, IN THE PRESENCE OF DIPHENOLS

(*R* = ratio of optical densities at 276 mμ : 250 mμ)

Diphenol	*R (in acid)*	*R (at pH 7)*
None	1 9	1·75
0 0002 per cent Hydroquinone	2 25	
0·0002 per cent Pyrocatechol		2 2
0 0002 per cent Resorcinol		2 3

One part by weight of diphenol per 100 parts of insulin was necessary. Similarly, in 20 per cent dioxan solutions there was no decrease in the value of *R*.

Solubility

In acetate solutions at pH 5, Lens (1948) obtained solubility curves typical of several components. This author thought that dilute acetic acid produces a slight decomposition of insulin. A purified batch gave a horizontal curve when the crystals were directly suspended in the acetate solution. *Fredericq and Neurath (1950), using sodium chloride,* also obtained horizontal solubility curves with a Lilly sample, lot T-2344.

The passage from acid solution to neutrality produces also a marked and irreversible decrease in the value of R. This

Table II

VALUE OF R FOR NORDISK SAMPLE AT VARIOUS CONCENTRATIONS

(R = ratio of optical densities at 276 mμ : 250 mμ)

Percentage concentration	R
0 2	2·48
0 1	2·38
0·05	2·25
0·01	1 9

decrease is stronger in dilute solution (Fig. 2, curve 1). In a 0·05 per cent solution, R changes from 2·25 (at pH 3·5) to

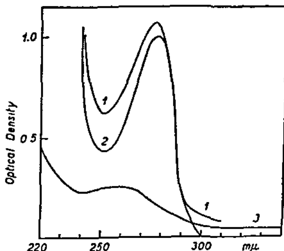


FIG 2 Absorption spectra of insulin solutions.

2·10 (at pH 6·7); after 24 hours standing at that pH it fell to 2·0. The decrease is greater at pH 7 and becomes less

all-glass tubes for 6 hours. A small amount of pyrocatechol (0·0002 per cent) was added to the buffer. After 30 minutes of centrifugation at 14,000 r.p.m., the concentrations of the supernatants were estimated from the optical densities at 276 mμ. The curves obtained with Boots, Lilly and Nordisk samples are shown in Fig. 3. All are typical of several components with miscibility in the solid phase. No plateau could be reached in the range investigated. The Nordisk sample was markedly more soluble.

For a given sample the solubility depends very much upon the nature of the salt medium. In Table IV are shown the solubilities of a Lilly sample at pH 7·5, the total insulin content being 2 g. per litre, and the salt 0·1 N.

Table IV

SOLUBILITY OF LILLY SAMPLE AT PH 7·5

Salt medium	*Solubility*
Sodium phosphate	soluble
Sodium thiocyanate	soluble
0 08 N-potassium chloride and 0 02 N-phosphates	0·15 per cent
Ammonium sulphate	0 12 per cent
Ammonium acetate	0 08 per cent
Sodium pyroborate	0 02 per cent

These results indicate specific interactions of insulin with anions, which are unexpected at this pH.

Fractionation of insulin

We think that a fractionation based on solubility should take into account the following rules:

1. Since the components are certainly very similar and capable of mutual interactions, it should be carried out at low protein concentration. Extraction procedures will be better than precipitation because in the former the most soluble molecules are first eliminated from the system.

According to our present data, acid media are not the most suitable for differentiating closely related components in insulin; it seems that solubilities are more spread on the alkaline side of the isoelectric point. On the other hand, the crystalline state of the solid phase is of paramount importance. For instance, insulin crystals are practically insoluble at pH 4–7; even at pH 8 a considerable amount is insoluble, and remains so when again equilibrated with new amounts of

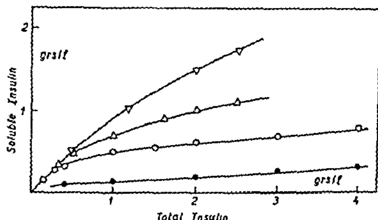


FIG. 3. Solubilities of insulin samples as a function of the total insulin.

● Lilly 535864 *pH 6·7*
○ Lilly 535664 pH 7·1
△ Boots pH 7·1
▽ Nordisk pH 7·1

solvent. A fractionation based on that property has been described previously (Fredericq, 1954). If the insulin crystals have first been dissolved in acid and reprecipitated, they will dissolve easily when brought to a pH greater than 6. In all solubility studies it is very important to check the crystalline nature of the solid phase under the microscope, particularly in samples that have first been completely dissolved. Since crystals are more readily obtained between *pH 5·5 and 7·5*, this range is the most suitable for the setting of solubility curves. Different *amounts of insulin crystals* were suspended in phosphate buffers and rocked in a thermostat at 25° in

The following is a typical fractionation process: 100 mg. of insulin crystals are suspended in 25 ml. of buffer at pH 7·1, at 25°. The suspension is rocked in a glass tube for two hours. It is centrifuged for thirty minutes at 14,000 r.p.m. The precipitate is suspended in fresh buffer; the supernatant is adjusted to pH 5·5 and dialysed without stirring against distilled water. After two days the dioxan is totally eliminated and insulin is precipitated for the most part. It is centrifuged and dissolved in dilute acetic acid; a small insoluble residue is discarded. Finally, it is recrystallized by the method of Romans, Scott and Fisher (1940). In some instances it was necessary to add some zinc in order to get crystallization.

This procedure gives very low yields due to the dilution of the insulin solutions and to the residual solubility of the protein at the pH of precipitation. It must be considered more for its theoretical results than for its possibilities of practical application.

We distinguish three main fractions: (I) extracted at pH 7·1, (II) extracted at pH 7·4–7·6, and (III) the residue. All of them are identical with the original sample with respect to the crystalline shape.

The solubilities in 0·1 N-phosphate buffers at pH 6·8 were determined on fractions isolated from two different samples,

Table V

SOLUBILITY OF LILLY AND BOOTS SAMPLES AT PH 6 8

Solubility in g. per litre

	Lilly	*Boots*
Crude sample	0·06	0 15
Fraction I	0·4	0 35
II	0·2	0 24
III	0 06	0 15

Lilly and Boots, and are given in Table V, in g. per litre; the total protein content was 0·4 g. per litre.

2. The most favourable pH range will be on the alkaline side of the isoelectric point because solubility anomalies are more marked there. Practically, this will restrict working between pH 6 and 8 because of the risk of degradation at higher pH.
3. Care should be taken to avoid inducing in insulin the spectral modifications described above, since we do not know to what extent they change the protein molecules.

Since the last condition is best fulfilled in acid media, we tried *an extraction in acetate buffers starting from* pH 4·7 to pH 4·2. We did not get fractions with significantly different solubilities and we decided to work in the neutral range in conformity with the second rule.

Several extractions of insulin crystals in 0·02 N- and 0·1 N-phosphate buffers were undertaken. Unfortunately this procedure, in which insulin is stirred for a long time at neutral pH, produces a strong decrease in the absorption ratio *R*, even when working in a cold room. We could, however, obtain by this procedure fractions with different solubilities. In some instances one of them had a lower sedimentation constant in acid phosphate than the original sample. But the reproducibility was not good and we abandoned this procedure in view of the risk of degradation and oxidation. An interesting feature of this fractionation is that from a Lilly sample we obtained after ten successive extractions a residual fraction which retained the crystalline form of insulin but *had a completely different spectrum,* as is shown in *Fig.* 2, curve 3. The yield was too low for the characterization of this fraction in other respects.

Finally, we adopted a 20 per cent dioxan solution, 0·05 N-phosphate, as a solvent for extraction. In this medium the spectral transformation is almost completely inhibited. On the other *hand, it was hoped to take advantage of the lowering* of the dielectric constant for the separations. We have already shown (Fredericq, *1953a*) *that dioxan promotes the reversible* dissociation of insulin. The protein is quite stable in those solutions and can easily be recrystallized from them.

pH 2·7. This corresponds to a molecular weight approaching 6,000. Since that time all samples investigated under those conditions gave higher values of S_{20}, generally around 1·6. Our Boots sample has $S_{20} = 1 \cdot 65$; for Fraction I, $S_{20} = 1 \cdot 4$, whereas for II and III it is 1·6. With the Lilly sample there is no lowering of S_{20} in any fraction, but the value for the crude sample is already low (1·45). These findings prove that part of the insulin molecules can undergo a dissociation into sub-units of molecular weight 6,000 in acid solution but that the dissociation constants vary according to the nature of the sample, the way in which it has been treated, etc.

Conclusions

Insulin presents a typical case of what has been named microheterogeneity (Synge, 1943; Colvin, Smith and Cook, 1954). There is no doubt that the particles of our insulin solutions are not identical chemical individuals. It is well established that they differ in size, in consequence of the reversible equilibrium of dissociation. But it appears also that the equilibrium constants are not the same for all the particles, and, therefore, that the elementary sub-units are different.

It is clear from Sanger's work (Sanger, 1952) that the differences do not concern the nature of the amino acids or their sequence in the polypeptide chains. They could arise from (1) slightly different physical configurations of the chains; (2) differences in the chemical groupings, or (3) strongly bound impurities. An answer to the first hypothesis will be given by the study of the molecular models of insulin which is now in progress in several laboratories and about which we shall hear more at this Colloquium. The existence of heterogeneity due to the second hypothesis has been proved by the work of Harfenist and Craig (1952). Finally we must consider the interactions of insulin with ions in solution, with particular reference to the rôle played by zinc.

The ease with which changes are induced in the physical properties of insulin during mild purification processes shows that only very superficial modifications are involved. We

In view of the marked differences in solubility, rough estimations of the zinc content were made by dithizone. It was found that Fraction I had a very low zinc content (about 0·1 per cent), II a slightly higher one, and III had practically the same zinc content as the original sample (0·5 to 0·6 per cent). Cohn and co-workers (1941) have shown that, below pH 6, insulin crystals have a zinc content which is quite constant and independent of the amount of zinc in solution; above pH 6, the zinc content increases only slightly. On the contrary, Hallas-Møller, Petersen and Schlichtkrull (1952) claim to have isolated crystals with widely differing zinc content between pH 5 and 7, depending on the total zinc in solution. We confirm here the finding of the latter authors that higher zinc percentages are associated with lower solubilities; but contrary to them, all our fractions were crystallized at the same pH, i.e. between 5·8 and 6. Craig (1953) found no difference in the zinc content of the A and B components. The possibility of an uneven distribution of zinc in insulin molecules certainly deserves further investigation.

The sedimentation patterns of Fractions II and III of the Boots sample are shown in Fig. 1 *g*, *h*. Fraction II is essentially depleted of the heavy component but still has a considerable spreading tendency; III is much more heterogeneous and enriched in heavy component; I is similar to II, although a little more heterogeneous. Other results from the Lilly sample confirm the finding that II has the most symmetrical boundary. It seems relatively easy to get rid of the heavy components by recrystallization or fractionation. But these procedures increase the proportions of light components. The sedimentation constant at pH 8 is 3·1 for the two original samples. It rises to 3·35 for II, and to 3·5 for I. Values of S_{20} ranging from 3·1 to 3·5 have actually been reported in the literature (see Creeth, 1953).

Marked discrepancies exist in the values of the molecular weight of insulin in acid solution (reviewed by Li, 1954). Using a Lilly sample, we found a few years ago a value of S_{20} = 1·3 for 0·25 per cent insulin in 0·1 N-phosphate buffer at

[illegible]

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DISCUSSION

Sanger: I wonder if these differences could be due to the differences in aggregation of the molecule, and whether the dimer and the monomer could separate from one another That might happen if there is a rather slow equilibrium

Fredericq: It is true that the existence of particles at different states of aggregation will produce an appearance of polydispersity in sedimentation patterns But if the samples were pure, the patterns should be identical in all cases investigated. On the other hand, they should not vary from one fraction to another.

It seems that for at least part of the insulin molecules, the equilibrium is rapid, since in some samples there is only one sedimentation peak. In other cases two peaks are observed; so we may have two kinds of particles, one with a rapid equilibrium and one with a slow one. As regards the supposed oxidation of tyrosine, we never got a reversibility of the spectral modifications, neither in the dilution effects nor in the pH changes.

[illegible]

favour the view that the heterogeneity is due to differences in the nature of a few chemical groupings (for instance, in amide groups, as found by Harfenist, or in tyrosyl residues as evidenced by spectra). Such differences will bring about variations in the charge and in the bonds between protein molecules and small ions. It is impossible to say now whether these variations occur during the preparation process or whether they pre-exist in the cells, in conformity with the general conclusion that Colvin, Smith and Cook (1954) drew from their critical study of the microheterogeneity of proteins.

As regards the prospects for fractionation, we think that there is only a slight possibility that great improvements could be made in the activity of insulin samples. We have not yet assayed our fractions, but from all the data which we have at present it seems that the high value now reached of 27 units per mg. will hardly be increased by further purification. From the physical chemist's point of view, it is nevertheless very important to pursue the study of insulin purification. From the experiments described in this work, we must conclude that the ordinary methods of fractionation based on precipitation or extraction are insufficient and that new and refined methods will be needed, together with a thorough knowledge of the stability conditions.

Acknowledgement

We gratefully acknowledge grants from the Lilly Research Laboratories, Indianapolis, and from the Centre national Belge de Biologie Physico-chimique.

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770 transfers. Obviously the further the distribution proceeded the more nearly a homogenous band of the main component was approached. Material from the main band was called the A component, that from the next most abundant band was called the B component.

A fair number of insulin preparations from other sources have been similarly studied. All showed the main component and varying amounts of the B component. In addition there were other components present in smaller proportions. We do not know the nature of these fractions but some of them had biological activity. Presumably they represent other insulins or insulin transformation products present in small proportions The A and B components crystallized readily and showed similar biological activity.

When Dr Harfenist (Harfenist, E. J. (1953), *J. Amer. chem. Soc.*, 75, 5528) made complete amino acid analyses on the purified fractions she found them to differ only as regards the amide groups. The A component showed six amide groups per molecular weight of 6000, while the B component showed five. Moreover, these analytical results were entirely consistent for the first time with a molecular weight as low as 6000. The previous analytical work from various laboratories had not been consistent for every amino acid residue with this molecular weight.

Insulins from swine and sheep gave similar distribution patterns and both were found to contain A and B components. Amino acid analyses showed distinct species differences but the same relationship of the A and B components. Again whole molecular ratios consistent with the smaller molecular weight were found.

It is well recognized that purity studies are of fundamental significance and essential for any reliable structural study. Degradation studies do have a bearing on the final concept of purity, but they are at best [illegible]

[illegible]

the concept of a molecular weight of 6000 presented at a time when there was general agreement from other sources that the molecular weight was 12,000.

[illegible]

the other? This would help to answer the question as to whether a configurational change might hamper part of the reaction. There is a possibility that one would have molecules which were relatively stable, say at pH 7, but if one were to take these and put them through an acid-alcohol or an acidic treatment, one would change the structural distribution. On going back again to pH 7 there would emerge two different kinds of molecules which would differ in their three-dimensional structure.

Craig: I would like to discuss briefly some purity studies of ours made during the past few years by countercurrent distribution. These studies show a number of impurities in every sample of insulin studied. From

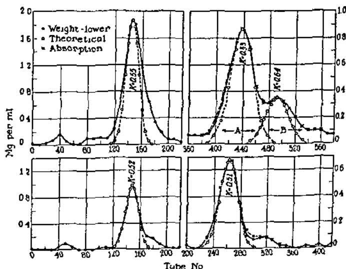


FIG 1 (Craig)

the data, it is easy to derive the percentages [illegible]

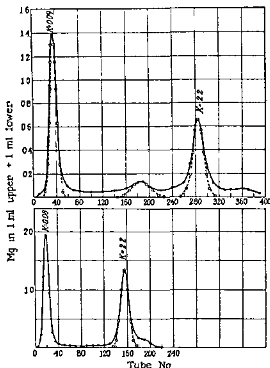


FIG. 3 (Craig).

main right-hand band, the solute crystallized directly. There has not been time to finish this work, but the crystals amounted to at least 25 per cent of the band.

respectively are shown. The starting mixture was made from 150 mg. each of swine and beef insulins. The purity of each starting sample was in the range of 90-95 per cent and it was not expected that only two completely pure bands would be obtained. The divergence from the calculated is about that expected. Irrespective of this it is obvious that the two insulins are separable by countercurrent distribution.

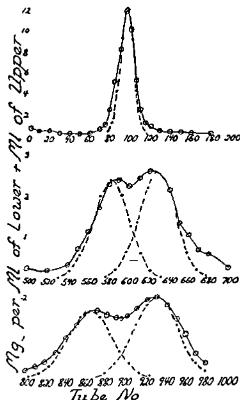


FIG. 2 (Craig)

important in considering the solubility

constant or whether there is a specific union with the dioxan in the solution or any other component of the solution.

Fredericq: We do not know, but we can expect that only by the lowering of the dielectric constant will there be an increase in dissociation, since we suppose the dissociation to be due to electric repulsion between particles, and the coulomb forces are stronger when the dielectric constant is decreased.

Craig: My point is that this effect could be due to the fact that insulin prefers to form a complex, insulin-dioxan, rather than an insulin-insulin complex.

Waugh: If one were to mix beef and pork insulin, and hold the mixture at a pH of 1·5, where the equilibria are rather rapid, then one should get about one-quarter of the dimers—this is the 12,000 units—as pork dimers, a quarter as beef dimers, and a half as a mixture of the two. If now, going back to pH 7 fixed the structure of the system, then on

Waugh: That is quite true. The physical studies under these conditions have not been carried out, this is part of the problem

Hodgkin: I am particularly interested in the fact that the B chain should crystallize in this way. Concerning the curves shown for the mixture of pork and beef insulin, perhaps under these conditions you

conversion of a monomer to a dimer to a trimer may be a function of the system, and in fact such could be an explanation of some of the heterogeneity that Dr. Fredericq had observed. I do not think there is any

the dissociation.

Young: I was wondering about Dr. Craig's point as to whether you would expect equilibrium to be quickly established under conditions of dimerization or polymerization of the 6000 unit?

Craig. I would expect it to be very quickly established, because reasoning from the rate of establishment of an equilibrium from the smaller peptide molecule there is no indication in all the work we have

established very quickly.

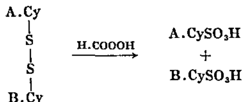
solutions. We found a complete dissociation into 6000 units near pH 4, under similar conditions, in water solutions, insulin is markedly aggregated.

Craig: Of course, one does not know whether it is due to the dielectric

from enzymic hydrolysates in which these groups remained intact (Sanger, Thompson and Kitai, 1955). Their position is also shown in Table I by the symbols $\underset{|}{NH_2}$.

The Disulphide Bonds

The sequences were determined on the separated polypeptide chains after breaking the disulphide bridges by oxidation, the cystine residues being converted to cysteic acid residues. The remaining problem was then to determine the distribution of the disulphide bridges in the original insulin. To do this it was necessary to subject intact insulin to partial hydrolysis and to isolate cystine-containing peptides, in which another amino acid was linked to each half-cystine residue. Such peptides could then be converted to two cysteic acid peptides by oxidation:



The cysteic acid peptides had already been encountered in the work with partial hydrolysates of the oxidized fractions, and their positions of origin in the polypeptide chains were known. It was thus possible to determine which half-cystine residues were joined together.

Initially the insulin was hydrolysed with concentrated hydrochloric acid, the standard reagent for partial hydrolysis and after rather extensive fractionation several cystine-containing peptides were obtained. Theoretically it was only necessary to identify two suitable peptides to determine the distribution of the three disulphide bridges, and from the first two peptides detected a structure could be deduced. However, when further results were obtained they did not fit in

THE CHEMICAL STRUCTURE OF INSULIN

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PREVIOUS work (Sanger, 1945, 1949*a*) has shown that cattle insulin (molecular weight 5734) is composed of two polypeptide chains joined together by the disulphide bonds of three cystine residues. The amino acid sequences in these two chains were determined by end-group studies and by partial hydrolysis methods (Sanger 1949*b*; Sanger and Tuppy, 1951; Sanger and Thompson, 1953) and are shown in Table I. The

Table I

THE STRUCTURE OF FRACTIONS A AND B OF OXIDIZED INSULIN

Chain	Sequence
Glycyl chain or Fraction A (Sanger and Thompson, 1953)	Gly Ileu Val.Glu.Glu(NH_2).$CySO_3H$.$CySO_3H$.- (1 2 3 4 5 6 7)
	Ala Ser.Val.$CySO_3H$.Ser.Leu.Tyr.- (8 9 10 11 12 13 14)
	Glu(NH_2).Leu.Glu.Asp(NH_2).Tyr $CySO_3H$.Asp(NH_2) (15 16 17 18 19 20 21)
Phenylalanyl chain or Fraction B (Sanger and Tuppy, 1951)	Phe.Val Asp(NH_2).Glu(NH_2).His Leu $CySO_3H$.Gly - (1 2 3 4 5 6 7 8)
	Ser His Leu.Val.Glu.Ala Leu.Tyr.- (9 10 11 12 13 14 15 16)
	Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.- (17 18 19 20 21 22 23)
	Phe.Phe Tyr.Thr.Pro.Lys.Ala (24 25 26 27 28 29 30)

distribution of the six amide groups on the glutamic and aspartic acid residues was determined by studying the ionophoretic mobility and amide content of peptides obtained

glycyl chain (positions A6, 7). It was therefore necessary to reinvestigate the possibility of carrying out hydrolysis in acid without disulphide interchange occurring, and it was eventually found that the reaction in acid solution could be inhibited by —SH compounds in marked contrast to the neutral reaction which was catalysed by —SH compounds. Thus under special conditions it was possible to obtain an acid hydrolysate of insulin containing cystine peptides which were in fact unaltered fragments of the original molecule. Although there were relatively few cysteic acid peptides obtained from the isolated chains, there were considerably more cystine peptides, especially of the type containing the CyS.CyS sequence, since such peptides contained fragments of three sequences, each of which could vary. It was thus necessary to carry out rather extensive fractionations, mostly by paper ionophoresis, before sufficient peptides of the "right type" could be obtained. Only a limited number of the peptides were in fact of the right type, for neither those containing the CyS.CyS sequence nor those giving a free cysteic acid residue on oxidation gave the required information about the distribution of —S—S— bonds. The only useful ones were those that gave two cysteic acid peptides on oxidation. In Table II

Table II

CYSTINE PEPTIDES IDENTIFIED IN PARTIAL ACID HYDROLYSATE OF INSULIN

Tyr Cy S S Leu.Val.Cy	Val Cy S S Glu.Cy	Ser.Val Cy S S Glu Cy	Cy Gly S S Cy.Ala
Leu.Cy S S Cy Ala	Leu Cy.Gly S S Cy.Ala	His Leu.Cy.Gly S S Cy.Ala	

are listed some of the cystine peptides obtained in this way. From their structure it can be seen that a disulphide bond joins the two chains together by the half-cystine residues in positions A7 and B7 and that there is an internal disulphide-containing ring in the glycyl chain between positions A6 and

with this structure, and in fact it seemed that every half-cystine residue was joined to every other half-cystine residue. This effect was shown to be due to a reaction causing a rearrangement of the —S—S— bridges which may be formulated as follows:



$$R^1SSR^1 + R^2SSR^2 \rightleftharpoons 2R^1SSR^2$$

Considerable difficulty was encountered in finding conditions for hydrolysis of insulin and fractionation of the resulting peptides under which this reaction did not take place, since it occurred both in acid and neutral solution (Ryle and Sanger, 1955).

The neutral reaction proved to be an —SH catalysed reaction and could be prevented by the addition of —SH inhibitors. It was thus possible to carry out hydrolyses with enzymes under conditions where the —S—S— bonds remained intact. Separation of the peptides was carried out by high-voltage ionophoresis on paper (Michl, 1951), which was found to be a very effective and mild method for fractionation of the relatively large peptides produced.

In this way a peptide was obtained from a chymotryptic hydrolysate of insulin which after oxidation gave rise to two cysteic acid peptides. These could be identified from their composition since they had been encountered in the chymotryptic hydrolysate of the separated chains. They were $CySO_3H.AspNH_2$ and $Leu.Val.CySO_3H.Gly.Glu.Arg.Gly.$-$Phe.Phe$. The former is the C-terminal dipeptide of the glycyl or A chain and contains the cysteic acid residues from position A20 and the latter contains the cysteic acid residue from position B19 of the phenylalanyl or B chain. Thus it was shown that a disulphide bond connects these two positions and holds the two chains together. Further evidence for this bond was obtained from several other peptides from both enzymic and acid hydrolysates. However, it was not possible to locate the remaining two —S—S— bonds using enzymic hydrolysates since no enzyme could be found that would split the bond between the two adjacent cysteic acid residues in the

found will be in parts of the molecule that are not concerned with the activity.

All the above work was carried out on cattle insulin. When insulin from pig or sheep was oxidized the products could be separated into a phenylalanyl and a glycyl chain with similar properties to those obtained from the cattle material, indicating a similar overall general structure for the three insulins. However, there were certain qualitative differences in the amino acid composition of the glycyl chains. The most obvious of these was the presence of threonine in the pig material compared with its absence in cattle and sheep glycyl chains (Sanger, 1949*d*). Harfenist and Craig (1952) carried out complete amino acid analyses of the three insulins and showed differences in their contents of serine, threonine, glycine, alanine, valine and isoleucine, which are summarized in Table III. The values for the other amino acids were identical.

Table III

AMINO ACID COMPOSITION OF DIFFERENT INSULINS
(Harfenist and Craig, 1952)

Results are given to the nearest whole number as amino acid residues per molecule of insulin

Amino acid	*Cattle insulin*	*Pig insulin*	*Sheep insulin*
Serine	3	3	2
Threonine	1	2	1
Glycine	4	4	5
Alanine	3	2	3
Valine	5	4	5
Isoleucine	1	2	1

In order to determine in which positions in the polypeptide chains these differences occurred the oxidized fractions from the two insulins were subjected to partial hydrolysis and the peptides produced compared with those obtained from cattle material (Brown, Sanger and Kitai, 1955). In this work it was not considered necessary to determine the whole of the sequences unequivocally, but rather to identify characteristic small peptides representing each position in the protein chains. Where these were the same as those from cattle insulin it could

A11. This structure is summarized in Fig. 1 (Ryle, Sanger, Smith and Kitai, 1955). The disulphide ring is of particular

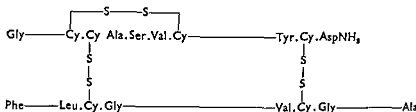


FIG. 1. The distribution of the disulphide bridges in insulin.

interest since it is of the same size as that found in the posterior pituitary hormones oxytocin and vasopressin.

Species Differences

The most interesting and important feature of insulin and, in fact, of most proteins is their unique physiological activity, and it must be assumed that this activity is dependent on their chemical structure. Studies on the effects of various chemical reagents on insulin (see Sanger, 1949*c*) have indicated that methods which bring about a definite disruption of the molecule, such as hydrolysis or reduction, lead to loss of biological activity, whereas certain other reagents which cause substitution or blocking of particular groupings such as amino groups frequently do not affect the activity. It would thus seem probable that insulin activity is determined by the specific arrangement of some amino acid side chains on the molecule to form an "active centre", that the intact structure of polypeptide chains and disulphide bonds is necessary to hold these groupings in their specific configuration, but that many of the side chains are not in fact involved in determining the activity.

One approach to the problem of the relationship between structure and activity is to investigate the insulins obtained from different animal species. These show the same biological activity (Scott and Fisher, 1940), so that any differences

and the amino acids present identified by paper chromatography. Again the results were the same for the pig and sheep as for the cattle material. Peptide 13, for instance, contains the amino acids [Thr,Ala,Tyr,Pro,Lys], and clearly corresponds to the peptide Tyr.Thr.Pro.Lys.Ala (positions B26–30) found in cattle material, since three of the amino acids, threonine, proline and lysine, occur only once in the molecule. This is a characteristic small peptide containing one residue of each of five amino acids, so it could be concluded that the five positions B26–30 are occupied by the same residues in cattle, pig and sheep insulin. Peptide 7 from the peptic hydrolysate (Fig 2) also contained the same amino acids as the corresponding peptide Tyr.Leu.Val.CySO$_3$H.-Gly.Glu.Arg.Gly.Phe from cattle insulin, but since it was relatively large it was considered necessary to subject it to partial acid hydrolysis and to identify small peptides in order to show that it also had the above structure. The peptides identified in this way are listed in Table IV. All of them fit

Table IV

SMALL PEPTIDES OBTAINED FROM PARTIAL ACID HYDROLYSATE OF PEPTIDE 7 (Fig. 2)

Structure of peptide 7 from cattle insulin	Tyr.	Leu.	Val.	CySO$_3$H.	Gly.	Glu	Arg	Gly	Phe
Peptides obtained from partial acid hydrolysate of peptide 7 from pig and sheep insulins			Val	CySO$_3$H		Glu	Arg		
				CySO$_3$H.	Gly		Arg	Gly	
					Gly	Glu		Gly	Phe
		Leu.	Val	CySO$_3$H					
			Val	CySO$_3$H	Gly				
	Tyr.	Leu.	Val.	CySO$_3$H					

into this structure, and since each position is represented by a small peptide it could be concluded that the sequence is present in all three insulins. From the study of further peptides from the peptic and from a tryptic hydrolysate in this way it was shown that in fact the complete sequence of the phenylalanyl chains is the same in cattle, pig and sheep insulin.

When the oxidized glycyl chain is hydrolysed with pepsin, splitting occurs at the Leu.Tyr bond (position A15, 16). All

be concluded that the position concerned was occupied by the same residue. Similarly, differences in the peptides indicated differences in the sequences which could be studied further.

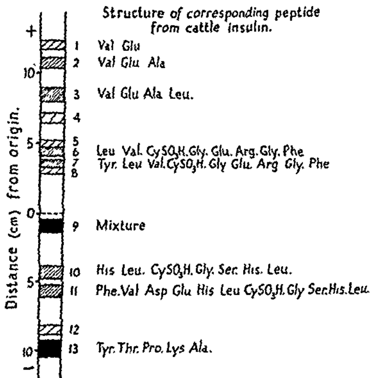


FIG. 2 Ionophoretic separation of peptic hydrolysate of Fraction B of cattle [illegible]

In the case of the phenylalanyl chain, the oxidized fraction *B* was first subjected to hydrolysis with pepsin and the peptides separated by paper ionophoresis. The resulting distribution of peptides (Fig. 2) was the same for all three species, suggesting that these chains were at least very similar if not identical. Each of the peptides was hydrolysed

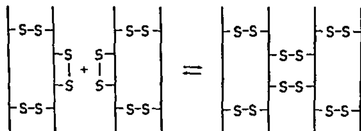
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DISCUSSION

[illegible]

figures.

Fredericq: Is there not a possibility of an equilibrium between 6000 and 12,000 molecules, according to the following scheme?



This would be an interchange reaction of disulphide groups similar to what you describe in your hydrolysis experiments. We would have to assume that according to the nature of the solution, the equilibrium could be shifted in either way.

[illegible]

peptides derived from the C-terminal side of this bond (positions 16–21) were the same for all three species, but differences were found in the peptide Ap5, which in the case of cattle insulin has the structure Gly.Ileu.Val.Glu.Glu.$CySO_3H$.-$CySO_3H$.Ala.Ser.Val.$CySO_3H$.Ser.Leu. A characteristic peptide, obtained in high yield by partial hydrolysis of cattle Ap5, is Ser.Val.$CySO_3H$. This was virtually absent from the pig material and in its place was found Ser.Ileu.$CySO_3H$, indicating that the valine residue found in position A10 in cattle insulin is replaced by an isoleucine residue in the pig insulin. Similarly, that the alanine residue in position A8 is replaced by a threonine residue in the pig material was shown by the absence of $CySO_3H$.Ala and the presence of $CySO_3H$.-Thr and Thr.Ser.Ileu.$CySO_3H$ in the pig Ap5. In this way it was shown that the only differences in the three insulins were found in the residues in the sequence A8–10, which in the cattle insulin is Ala.Ser.Val, in the pig Thr.Ser.Ileu, and in the sheep Ala.Gly.Val. The rest of the molecule is identical in all three species. It is interesting that these differences are found in the residues that are included in the disulphide ring and that therefore the exact structure of this part of the molecule is not essential to the physiological activity of insulin.

This work was carried out in collaboration with A. P. Ryle, L. F. Smith, R. Kitai and H. Brown, and will be published in detail elsewhere (Ryle and Sanger, 1955; Ryle, Sanger, Smith and Kitai, 1955; Brown, Sanger and Kitai, 1955).

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[illegible]

do not think you could get an intelligent answer, making these simple assumptions.

Craig: With some of the bonds you could.

Sanger: I agree with Dr. Craig that we must consider these possibilities. The spectrum changes might suggest something of that type.

Porter: Do you think that the whole structure is necessary for activity? Is there any evidence of activity surviving partial degradation?

Sanger: So far it has been almost impossible to hydrolyse insulin without destruction of its activity. The only thing which has been possible is to split off the alanine from the C-terminal end of the phenylalanyl chain. That appears to give, according to Harris and Li, an active substance, but any further treatment with proteolytic enzymes

[illegible]

is no evidence of any binding of the coloured substance to the insulin, and if that disulphide bond were reactive it should react with the bis-DNP cystine. Cystine itself does react very rapidly, but insulin does not, so that the disulphide bonds do seem to be very stable to this interchange reaction.

weight has been estab

chloride (Kupke, D.

formula we have deduced (the upper one in Fredericq's diagram).

Craig: You mean the interchange would be rapid enough to fit in with these findings? I think the possibility a little far-fetched.

Sanger: So do I.

Waugh: I hope to show shortly that simple dilution at pH 2 will dissociate insulin into the 6000 unit.

Fredericq: Have you ever found in your hydrolysates any evidence for the presence of oxidized tyrosine?

Sanger: There is evidence derived from the performic acid oxidation

Fredericq: Do you find also the same type of spectra with an increase at the minimum absorption?

eliminated

evidence that the unit of 6,000 was most probable lay in the dissociation observed by Harfenist and Craig (1952) and by Kupke and Linderstrom-Lang (1954). Since either chemically modified insulins or insulins placed in high concentrations of guanidinium ion were used in these experiments, it is of some interest to report that Dr. David Yphantis and I, using a separation cell technique which allows us to make molecular

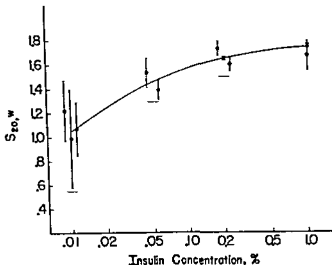


FIG. 1. Sedimentation coefficient versus concentration of insulin

weight determinations at very low concentrations (to be published), have found that, at pH 2·5 and $\Gamma/2 = 0 \cdot 1$, insulin dissociates into units less than 12,000, the predicted minimum being 6,000. The situation is shown in Fig. 1, which plots sedimentation constant versus concentration.

The sub-units of $M \sim 6{,}000$ combine strongly to form stable dimers, which are the smallest units ordinarily observed, as for example in the experiments of Gutfreund (1952), Fredericq (1953), Tietze and Neurath (1952, 1953), Oncley

A CONCEPT OF THE THREE-DIMENSIONAL STRUCTURE OF INSULIN

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Considerable attention has been given within the last few years to the geometrical arrangement of the main chain of proteins. To an extent the advances which have been made in this direction owe their origins not only to the fact that the sequence of atoms —CHNHCO— repeats in large measure in a sterically uniform manner, but also to the fact that the NHCO group is coplanar (Corey and Pauling, 1953). When coupled with the assumption that a maximum number of appropriate hydrogen bonds must form, there emerge, as have been described by Pauling and Corey (1953), a limited number of folds, several in the form of helices.

We can have little doubt that the side chains also play an important part in determining the structure and properties of proteins. At the same time their diversity of size, structure, and chemical properties suggests that the set of rules governing the side-chain behaviour will be more complicated than the set related to the main chain. For purposes of examining the rôle which side chains play in defining structure, insulin offers an unusual opportunity.

We have found, in the preceding discussion, answers to questions concerning the sequences of amino acids in the A and B chains of insulin. Dr. Sanger and his colleagues have also specified the positions of the amides of glutamic and aspartic acids and have given in detail the positions of the disulphide links. It was, of course, possible that linkages through the same sequences of amino acids might lead to covalently bonded units of 12,000 rather than 6,000; the

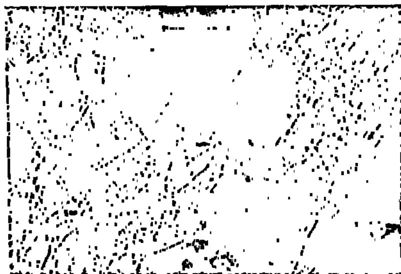


FIG 2. Electron micrograph of insulin fibrils shadowed with chromium The scale line indicates 1 micron

and co-workers (1952), Doty and co-workers (1952, 1953) and Steiner (1952, 1953). In these experiments it was found that a set of readily reversible equilibria exist between the M = 12,000 units (the effective monomer, which will be referred to as M12) and dimers, trimers, and quadrimers. The equilibria are sensitive to pH and ionic strength in such a way as to suggest that dissociation is due to electrostatic repulsion between similarly charged particles. The energy required to accomplish association is probably due to short-range forces such as electronic Van der Waals forces and hydrogen bond, a situation which will be discussed below. It is significant that Doty and Myers (1953) and Steiner (1953) observe an entropy decrease on dimerization which, according to Doty and Myers, is about 110 E.U. *smaller than the entropy decrease predicted* on the basis that two structureless cylinders are associating. They suggest that the simultaneous freeing of 24 molecules of water of hydration would be sufficient to account for the entropy change observed.

When heated in solution at pH values below $\sim 3 \cdot 5$ native insulin spontaneously aggregates to form sub-microscopic fibrils. These are shown in Fig. 2. Insulin fibrils have, in contrast to the association systems just described, a remarkable stability, being stable at least from pH 0 to pH 10 (Waugh, 1948). The kinetics of fibril formation (Waugh *et al.*, 1953) suggest that a fibril is initiated by a nucleation reaction in which three or four monomers co-operate in forming a group as indicated diagramatically in Fig. 3 (based on an analysis by Koltun and co-workers, 1954). Not only does this group have the requisite stability but it provides a surface structure which can induce a single entering monomer to bond in a manner which conforms to and perpetuates the unusual structure of the group. The consequences of the co-operative effect are seen readily in the following seeding experiment. Fibrils formed at higher temperature are seeded into a solution of native insulin at a low temperature and at low pH. The fibrils will grow in length and diameter and remove all native insulin (Waugh, Thompson, and Weimer, 1950).

in the absence of the seeding fibrils the solution of native insulin would have been stable essentially for an indefinite time. We note that seeding conditions are similar to those in which reversible associations appear; yet the fibril perpetuates its own stable structure.

The structure of the insulin molecule in the fibril, although it may not be that of the molecule in solution, is preserved in

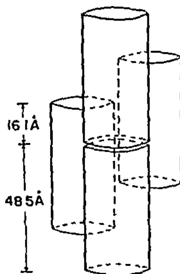


Fig. 3. Schematic representation of the way in which four effective monomers (M12) may establish a stable insulin fibril nucleus

its essential aspects, for fibrils will disaggregate at pH values near 12 (Waugh, 1948) and all of the protein can be recovered in a form identical with that of native insulin.

Questions concerning the compactness of the fibril will arise shortly. Momentarily, it is desirable to examine the nature of the interinsulin linkage.

This question has been approached in two ways (Waugh *et al.*, 1953). F[illegible]

proline and tyrosine, all of which are non-polar in character and considerably larger than average. Each large non-polar side chain has the equivalent of 4·8 CH_2 groups. Debye (1949) in his examination of the formation of soap and detergent micelles finds that each CH_2 group contributes an attractive interaction energy of 1·18 kcal. per mole. The energy differential arises from the formation of new hydrogen bonds through the disappearance of hydrocarbon-water interface (see Waugh, 1954). Thus the complete interaction of an average non-polar side chain with similar groups would yield an energy of 5·1 kcal. per mole residues. Each mole of insulin (M12) contains 43 moles of non-polar side chains. An interaction energy totalling over 200 kcal. per mole protein appears to be available. It is seen at once that the interactions of appropriate fractions of the large non-polar residues will lend considerable stability both to the structure of the monomer and to various types of interactions into which the monomer may enter.

Since an interaction of $\Delta F = -10$ to 20 kcal. per mole leads to linkages which are stable with respect to thermal agitation, we see that our problem is not one of seeking an appropriate source of linking groups but of accounting adequately for the fact that insulin, and incidentally most other corpuscular proteins, can exist in forms which are soluble even at pH values removed from their isoelectric points.

We may choose to approximate the structure of the effective monomer as indicated in Fig. 4. Here the small circles are the equivalent of the domain of the main chain, assumed to be an α-helix, and the large circles represent the average fully extended side chain. The main chain centre-to-centre distance is 10Å, as suggested by crystallographic data (Low, 1952, 1953). Clearly on this basis the larger side chains are bent, positioned and packed in some "accommodating" fashion. The wavy line of Fig. 4 suggests a demarcation between an internal volume and accessible surface regions. The nature and structure of the internal volume are of particular interest.

chemically modified insulins have been examined. These include completely esterified and acetylated insulins, and insulins coupled with diazonium salts in which azotolyl, azobenzene sulphonic acid, and azobenzoic acid groups were added. All of these chemically modified insulins form insulin fibrils. On the other hand, the disulphide bonds of insulin must remain intact to obtain fibrils. The second approach involved the use of solvents containing compounds capable of forming strong hydrogen bonds. It was found that fibril growth will proceed to completion in the presence of 8·3 N acetic acid, in similar concentrations of other organic acids, and in 5·3 to 6·0 M urea at pH 1·6 or pH 7·0. In the absence of urea, fibril growth will not take place at pH 7·0.

The circumstances of growth at low temperatures and in the presence of organic acids and urea, the fact that chemically modified insulins readily form insulin fibrils, and the fact that fibril formation is accelerated as the pH is decreased from ~pH 3·5, taken together effectively eliminate covalent bonds, electrostatic interactions, and hydrogen bonds as being the primary contributors to interinsulin linkage. The characteristics of fibril formation suggest that interactions on the part of the non-polar side chains of insulin would provide a linkage having the requisite properties; for example, a linkage stable in itself with respect to changes in pH and ionic strength. This conclusion is strengthened by some experiments with Dr. Walter Koltun (unpublished). The introduction of ten essentially non-polar azotolyl groups per M12 renders the insulin insoluble. The introduction of five groups leads to a protein having a sedimentation coefficient of ~10·0 at 0·5 per cent in 0·1 N-HCl; a peak having S ~2·4, characteristic of M12, appears only on extensive dilution. Also, this modified insulin yields fibrils which are not disaggregated in alkaline solution as are fibrils from native insulin.

We may use this information to approach the problem of structure. An examination of the side-chain composition of insulin reveals immediately that 43 per cent of the side chains are represented in the group valine, leucine, phenylalanine,

of the apparent molal volume, may represent the extent of unoccupied space. A similar small excess volume is clearly indicated from the enzymatic digestion of insulin (and other proteins) as detailed by Linderstrom-Lang (1949). The cleavage of the first few peptide bonds leads to a volume decrement in excess of that expected from the electrostriction of water by the charged groups which appear. This decrement is also about 3 per cent of the apparent molal volume. Linderstrom-Lang suggests that the protein molecule has some forced structure which makes it occupy slightly more space than the unfolded elements.

If we assume that all unoccupied space is in the region of the internal volume, the internal volume will have only about 10 per cent of voids. We see in this way that the side chains are closely packed in the internal volume. The fact that at least one end of each side chain is fixed by the structure of the main chain suggests two further important observations. First, the larger than average side chains must in general be fitted in with smaller than average side chains on neighbouring rods, and second, the non-cylindrical side chains must have little freedom to rotate around most of their single covalent bonds. We therefore expect a high degree of organization in the packing of the side chains and a low entropy.

The insulin fibril is also found to be a close-packed structure. By flotation in solvent mixtures of different density a value of 1·35–1·4 is obtained. This value is close to the reciprocal of the partial specific volume of insulin and suggests that the protein elements, including all spaces inaccessible to the solvent used in flotation, are close-packed—almost to the limits imposed by the Van der Waals radii of the atoms. Close packing, at least in regions including certain of the histidine side chains, is shown by the experiments of Porter (1950), who found that 3·6–3·7 out of 4 histidine residues in native insulin were reactive to 1 : 2 : 4-fluorodinitrobenzene, while, in the fibril, only 2·2 out of 4 were similarly reactive.

The situation may be somewhat different for insulin crystals. Here linking through charge interactions is indicated by the

The α-helix is the most compact form in which the main chain may be arranged, as is readily indicated by comparing the density calculated from the α-helix with that obtained by applying Traube's rule, in which we sum the volumes of the constituent groups and divide the mass by this volume. In both cases a density of 1·69 is obtained (see Waugh, 1954).

As side chains are interdigitated in the internal volume, unless packing is nearly perfect, we expect to find spaces. These spaces may be large enough to accommodate one or more water molecules, and will be filled with water if they are accessible to the solvent. If they are smaller than water molecules, or are not accessible to water, they will represent voids or unoccupied space. McMeekin and Marshall (1952) have made an examination of a variety of proteins, including insulin, and find that the partial specific volumes, $\bar{V}$, as calculated from Traube's rule, agree excellently with the measured values. One would expect, of course, that electrostriction of solvent molecules around polar groups would make the measured $\bar{V}$ smaller than that calculated from Traube's rule. The difference, amounting to about 3 per cent

small changes in the configurations of the interacting units may lead to large changes in interaction energy.

The purpose of the present discussion has been to outline the importance to be assigned to the side chains as components which establish structure and stability in the monomer and its association and aggregation products. Attention has been directed specifically to the larger non-polar side chains, which constitute one-third to one-half the side chains of globular proteins. Other side chains have been neglected for reason of time. For example, the side chain of tyrosine, when ionized, appears to play an important rôle in decreasing the stability of the insulin fibril.

Since the insulin molecule appears to be a relatively compact structure, the requirement that all space be essentially occupied suggests that large and small side chains occur in an appropriate three-dimensional pattern, which is dictated step-wise by the sequence in the polypeptide chain, the disulphide cross-links, and the folding or coiling of the chain.

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intervention in all cases of an additional interactant (zinc or sulphate) which has a charge opposite to that of insulin. At the same time we must suppose that the introduction of the additional interactant prevents close approach of the surfaces of small associations of the effective monomeric units of M12.

Our present view suggests that the structure of the (effective) monomer is stabilized partly through the positioning of non-polar residues so that they may interact effectively. On the other hand, a frequency of occurrence of 0·43 for large non-polar residues suggests that a highly efficient interaction of even a small fraction of the total number (at any stage of association or aggregation) will lead to an entirely stable linkage. For a two-rod structure of M6 it appears unlikely that more than a small fraction of the non-polar side chains are effectively utilized, and are therefore made unavailable for further interaction. Where we observe readily reversible associations the interactions of the larger non-polar residues must be made less energetic, either through steric effects which prevent close approximation or through endothermic side reactions (such as the removal of water of hydration) which occur concomitant to interaction. The stable aggregate represented by the fibril on this basis would require either a more effective interaction of groups normally responsible for reversible associations or in addition the recruiting of a few additional side chains.

We may now examine briefly the extent of structural alteration which will materially decrease the electronic Van der Waals interactions of non-polar or other similar types of bonds. In systems stabilized by a series of secondary valence forces the interaction energy decreases with the inverse sixth power of the distance of group separation. The distance of close approach corresponds to the sum of the Van der Waals radii, the average Van der Waals radius for most of the side chain groups being about 2·0Å. If the groups are separated by only one additional Ångström unit, the interaction energy decreases by approximately 75 per cent. Thus, relatively

A SPECULATION ON INSULIN

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DR. SANGER's deduction of the complete amino acid sequence in insulin (Sanger and Tuppy, 1951; Sanger and Thompson, 1953) and of the position of the disulphide bridges (Sanger, Smith and Kitai, 1954) invites speculation on the detailed configuration of the peptide chains. This speculation is helped by evidence, firstly, of the geometrical character of cystine; secondly, of both chemical and crystallographic studies of small peptides, and thirdly, of the X-ray crystallography of rhombohedral zinc insulin, acid insulin sulphate and fibrous insulin.

The molecule of cystine itself has in the crystal structure (Bernal, 1931) a twofold axis of symmetry, and this symmetry is shown also in the crystal structure of diglycyl cystine (Yakel and Hughes, 1954). If peptide chains have to be joined by disulphide bridges, two relative orientations, (*a*) and (*b*), are possible (Fig.1) in which the full twofold symmetry of the disulphide bridge is preserved. Chains situated in (*a*) are so arranged that they are hydrogen-bonded together, while in (*b*) the hydrogen-bonding direction is at right angles to that of the cystine bridge. It is possible, in both cases, to arrange that the two separate chains become part of one chain by turning one residue at X at right angles to the main chain direction. Then, in both cases, cystine bridges could form easily between residues exactly 5 apart in the amino acid sequence. This is the interval found in insulin, where residues numbers 6 and 11 are linked together in the A chain, and also in oxytocin (du Vigneaud, Ressler and Trippett, 1953; Tuppy, 1953) and vasopressin (Acher and Chauvet, 1954).

So far only the geometrical form of cystine bridge represented

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[Discussion of this paper was postponed until after the paper by Dr. Hodgkin.—ED.]

by (*b*) has actually been observed—in diglycyl cystine (Fig. 2); but that represented by (*a*) has certain definite advantages for disulphide bridge appearance within a single peptide chain since its formation might well be encouraged by the internal hydrogen bond system drawing the chains together.

We were first led to consider folding peptide chains in this way by observations on gramicidin S, which has been shown

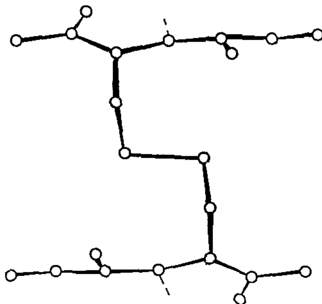


Fig 2 A view of the arrangement of the peptide units found in diglycyl cystine. Compare Figure 1 *b*

by a number of measurements to be a cyclic decapeptide (Consden *et al.*, 1947; Battersby and Craig, 1951; Belogorsky and Paskhina, 1945; Pederson and Synge, 1948). From a study of the rather complicated crystal structures shown by its derivatives, two facts emerge: firstly, that the molecule appears to have a twofold axis of symmetry, and secondly, that the atoms in the molecule tend to be concentrated in layers 4·8Å apart, at right angles to this axis. It is possible

FIG. 1. Two possible arrangements of peptide chains linked by disulphide bridges. X, residue placed to link chains together.

combined together as a coherent unit of molecular weight 6000; and two such units may easily fit, side by side, to build a compact molecule of molecular weight 12,000. Fig. 4 shows a possible model.

This may be too simple and unsubtle a form of molecule to suggest for insulin, but it has certain features which commend it. It is compact, of dimensions that would fit into the unit cells of both rhombohedral zinc insulin (Crowfoot and Riley, 1939) and acid insulin sulphate (Low, 1952). In zinc insulin the sheets would probably lie in a general direction perhaps parallel to the plane (1$\bar{4}$32). In acid insulin sulphate the strength of the (042) reflection suggests that this is the plane of the sheet and that the appearance of rod-like structures in the Patterson series calculated for the dry crystal might, at least in part, be an artifact of the limited X-ray data used. The most striking feature of the fibrous insulin pattern, the very strong 4·8Å meridional reflection (Waugh, 1954) (strongly reminiscent to us of the characteristic reflections observed in gramicidin S), could be explained as the distance between hydrogen-bonded chains within the sheets. It is noticeable that strong reflections of this spacing are also observable for zinc insulin.

In a formal way this insulin model can be derived from the antiparallel pleated sheet configuration proposed by Pauling and Corey (1951) for β-type proteins. The characteristic distance of about 4·8Å between chains is essentially the same as the backbone spacing of β-proteins usually quoted as 4·67Å—the slight lengthening, if real, may easily be accounted [illegible]

Of this, probably the most important is the spectroscopic evidence that the frequencies of the CO- and NH-stretching modes are those characteristic of α-folded proteins (Ambrose and Elliott, 1951). However, it is notable that these frequencies shift to the β-type in fibrous insulin, although Waugh and his co-workers are convinced that no major configurational

to build various peptide models which conform to these conditions; one of these (Fig. 8) contains the postulated fold, here formed by the proline residues known to be present in the molecule. This type of cyclic molecule can be constructed with six, ten or fourteen, i.e. $6 + 4n$, residues in the ring. It may be the merest coincidence that another cyclic peptide, phalloidin (Melrun, Keil and Sorm, 1954), on the chemical

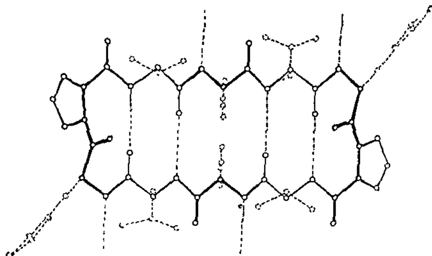


FIG. 8. Possible configuration of peptide chains in gramicidin S.

structure of which considerable evidence has accumulated, has a six-residue ring, and so belongs to this series.

The simplest way in which to use the (*a*) type of fold in constructing a model for insulin is to form separate sheets of the A chain and the B chain. These must be folded in such a way that the cysteyl residues at 7 and 20 come out on the same side of the A sheet, and residues 7 and 19 on the same side of the B sheet, at distances apart which would enable them to be linked together by disulphide bridges. This can be done in several ways if one of the two disulphide bridges is allowed to form in a stereochemically not unreasonable way but without full twofold symmetry. The two sheets are then

change within the molecule is associated with the transformation to fibrous insulin. One may therefore suggest that the frequency shifts are associated with some modification of the CO and NH bonds which occurs without major changes in the chain configuration. This modification should then also be present in gramicidin S, which, like insulin, has α-type spectroscopic characteristics (Abbott and Ambrose, 1953). The evidence may at least be judged conflicting.

It is also, most justifiably, arguable that in building a model of this sort we have followed far too rigidly the consequences of a piece of formal geometry. The stereochemical conditions determining the likely distribution of atoms in space are actually much less rigid than is suggested by the formal geometry of cystine. It is not particularly difficult to build a number of other models for insulin conforming, at least partially, to the conditions of other suggested modes of chain folding. For example, though it is not possible to form the disulphide bridge between residues at 6 and 11 within the α-helix fold of Pauling, Branson and Corey (Pauling and Corey, 1950; Pauling, Corey and Branson, 1951), it is quite conceivable that a major part of the insulin molecule conforms to the α-helix pattern. Detailed models have been built in several laboratories, and one, devised by Dr. Lindley and Dr. Rollett, is shortly to be published (Lindley and Rollett, 1955). In this model the α-helix sequence in chain B is broken between residues 6 and 11 somewhat in the manner illustrated in Fig. 5. Above and below the break the α-helix is wound in opposite directions, left-handed and right-handed respectively. The A chain, if wound left-handed in the α-helix form, can then be attached to the B chain by the remaining disulphide bridges. The two-chain model that results can readily be packed with the two chains of a second molecule to form a four-chain structure, the 12,000 unit, which it is possible to fit into the various crystals examined.

Model-building for insulin seems to us at present a pastime which, while interesting and suggestive, is unlikely to lead to definite conclusions for some time to come. It does give us,

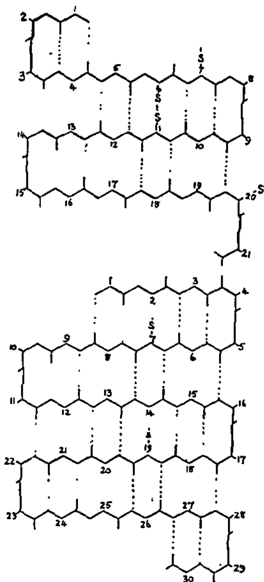


FIG. 4. Diagram to illustrate a possible method of folding the A and B chains of insulin into sheets.

of chain folding that it could lead us, step by step, to the precise distribution in space of all the atoms in the molecule.

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DISCUSSION

Hodgkin · Have you any explanation for the differences in your observations on fibril formation and reversal? What do you imagine happened to prevent the reversal to fibril formation under certain conditions?

Waugh. One can effectively prevent the reversion of a fibril, by methylating the carboxyl groups, for example. Here, as one increases the pH, no negative charges appear, so that repulsive forces are absent and the fibril remains quite stable under conditions where the native

however, one impression which is *encouraging*, that the molecule is quite small and, in a crystallographic sense, manageable, given the existence of Sanger's detailed chemical evidence. *Insulin in the crystals so far examined is eight* times larger in molecular weight than any molecule of which

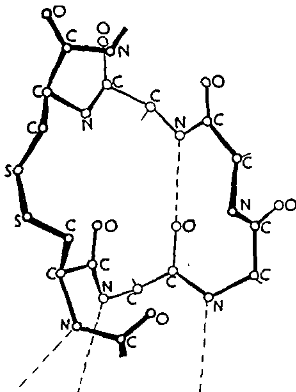


FIG. 5. Disulphide bridge within peptide chain coiled above and below the link in α-helices of opposite sense.

the crystal structure has yet been solved, but this means that, in each dimension, it is only about twice as great. If we could examine a series of derivatives of insulin in which different known residues were marked with heavy atoms we ought to be able to find the relative positions of these residues quite exactly. *This information should so limit the possible modes*

charged group which co-ordinates water-molecules. The co-ordination of water-molecules in this local region produces a rather dramatic volume expansion. To speculate further, this volume expansion just forces the surfaces of the proteins far enough apart so that the interaction

Waugh: The intervention of the zinc atom in the zinc insulin crystal or the sulphate group in insulin sulphate crystals might produce two effects: first, to define very precisely the positions where the monomers

cent, this represents an approximately 10 per cent change in the average group separation. This may not be big enough to let any water-molecules in, and yet in performing this expansion one must invest the Van der Waals attractive energy

Craig. There is one very interesting observation which Prof. Waugh has reported If insulin is converted to fibrils, then reconverted by bringing up the pH and crystallizing, it seems to be more easily converted to fibrils. If the conversion is repeated five or six times the crystalline insulin seems to be more unstable and goes easily over to fibrils. Is this correct?

Waugh. Yes.

Craig: How does this behaviour fit in with our concepts? Is it as if the substance has a memory, or is it just that some seeds remain? Could the latter explain the phenomenon?

Waugh Yes On heating, insulin will go into the fibrous form in a matter of 3 hours. Now, if one introduces just a few seeds into this solution at the same temperature, the fibrous form will develop in a

insulin fibril is unstable. The second system is to add to the tyrosine

down, the liberated insulin molecules break down.

Sanger: Prof. Waugh, have you any idea which particular groups they might be which are involved in forming the fibrils? It is a very specific reaction, isn't it?

Waugh: It is specific in some respects and not in others, e.g. it is specific in the sense that if one alters the disulphide groups, one no longer gets insulin fibrils. Apparently one disulphide linkage per molecule of 12,000 is sufficient to do this. Perhaps this represents some sort of interference effect between native and reduced units of 6000; but, remarkably enough, fibril formation is not specific with respect to chemical modifications, e.g. in introducing say ten azotolyl or azobenzene

with it or

to get an

Waugh: If this is a non-polar linkage there is rather a wide opportunity for interaction. I think possibly this accounts for the diffuseness of the X-ray diffraction patterns. For example, if one were to have specified points on each molecule which had to come together, the resulting pattern of molecules should have a rather perfect three-dimensional regularity. On the other hand, if the surfaces have a complexion of

the fibril. But phenol has a further most interesting effect. If one takes a system of insulin fibrils and adds phenol to give about 10–20 per cent

alkaline treatment, although again the structure of this new set of

G. Cori: Prof. Waugh, did you say that fibrinogen forms fibrils? How does this compare with fibrin?

Waugh: I wish that I could answer this important question. We have been studying the transformation of fibrinogen into fibrin through thrombin and we had a brief look at the fibrils which form under the influence of heat, and also under the influence of acid. We have not come to the point where we can make any comparison as to what is holding

point, is that possible?

Waugh. It is possible. This is the very line that we hope to pursue more.

C. F. Cori: Prof. Waugh, how did you arrive at the precise structure

three or four monomers (we think it must be four monomers). The

Sutherland: At one time we followed Prof. Waugh's instructions for [illegible]

the conditions identical?

Behrens: We have not had extensive experience with cross-seeding, but certainly under the conditions that produce insulin fibrils glucagon fibrils are also formed very well.

Craig: There is no sulphur, no cystine, in glucagon?

Behrens: Yes there is sulphur, but as methionine, not cystine.

Sanger: Does this come out with insulin?

Behrens. Partially, but this has not been done as carefully as we would like.

von Holt: Is it possible to form fibrils in the absence of zinc?

Waugh: Yes, all our studies on fibrils were carried out with insulin which has been electrodialysed to the point where dithizone does not detect the presence of zinc. It is for this reason also that I think that the 12,000 units is probably not stabilized by the intervention of the zinc atom.

Best: Prof. Waugh, are there any other protein hormones which form fibrils?

Waugh: We have not examined other protein hormones. We have examined serum albumin and it will form fibrils which can be taken apart again to give serum albumin or something which looks very much like serum albumin. We know this can be done also with fibrinogen.

Lens: When the terminal alanine group is removed from insulin by carboxypeptidase, one product is obtained which no longer gives fibrils. This proves that your method is very specific indeed. How does this fit into the picture? By just removing one single alanine a molecule is obtained which no longer forms fibrils.

Waugh: Chymotrypsinogen behaves as a monomeric system under certain conditions (references in Waugh, 1954). One activation product, α-chymotrypsin, under the same conditions exhibits a reversible dimerization. I feel that we require additional information, particularly information concerning the structural changes which follow removal of [illegible]

structure.

[illegible] explain the fact that these fibrils so freely dissolve in [illegible]

INSULIN AND GLUCAGON

WERNER SCHULZE
Medical Clinic, University of Leipzig

Development of glucagon research

About thirty years ago, after the successful experiments of Banting and Best, there was good hope that not only all the therapeutic, but also the pathogenetic problems of diabetes had been cleared up satisfactorily. The regulating influence of the pancreas on the carbohydrate metabolism seemed to be restricted to the action of a single antidiabetic hormone, and, accordingly, diabetes was at first thought to be due exclusively to deficient insulin production.

Yet, as early as in the next ten years, the study of the physiological action of pancreatic extracts and of insulin preparations, although still rather crude, yielded some findings which did not fit in with the classic theory of diabetes.

In 1923 Murlin and co-workers described a biphasic reaction of blood sugar in animals after injection of pancreatic extracts. Murlin interpreted the initial hyperglycaemic phase as an effect of an additional and hitherto hypothetical hormone of the pancreas, which he called "glucagon". Subsequently, that dualistic theory of the pancreatic incretion went unnoticed by the physiologists and was not followed up systematically even by Murlin himself.

In 1928 and the following years Bürger became aware of the glucagon problem when studying the physiology of insulin, and came independently to the same conclusions as did Murlin In view of the pioneer work of Bürger and his associates I should like in my presentation to review the course of his investigations. Bürger, as a disciple of Hofmeister, had obtained his biochemical training in the same laboratories as Mehring and Minkowski.

Fredericq: If we adopt any of the models for the insulin molecules,

not know.

Fredericq: From X-ray data could you not distinguish between these possibilities?

from the pattern of reflections. One can see whether some kind of model roughly begins to fit; this does not mean that the model is correct at all, far from it. But this is not to say that we may not know precisely one day.

[illegible] in the structures which you built on the basis

models suggested.

Hodgkin: I think it would be very interesting to do this

he had repeatedly observed the contrary effect, i.e. a progressive depletion of the hepatic glycogen store.

Burger agreed with Macleod that the skeletal muscles were the real site of insulin action, and thought that the specific physiological purpose of insulin was to promote sugar utilization in the periphery. That idea was based on his observation, with Kramer, of an abolition of the hypoglycaemic insulin effect following ligature of the large peripheral arteries in dogs. Figure 2 shows that the typical insulin effect does not appear during interruption of the peripheral circulation, whilst it reappears immediately the circulation is restored in the extremities.

Bürger's supposition proved correct in view of the favourable influence of insulin therapy on the uneconomical work of muscles in diabetics, which was confirmed in later investigations (see p. 260).

"Initial insulin hyperglycaemia" as the starting-point of Burger's glucagon research

The studies mentioned above led Bürger to the discovery in 1928 that intravenous administration of insulin produces an initial rise in blood sugar in dogs (Fig. 2). It was that seemingly paradoxical side effect that induced Burger to examine

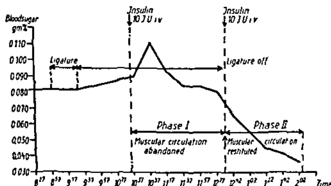


FIG. 2. Glugacon effect after ligature of the greater muscular arteries (pinching off the Aa. iliacae and subclaviae).

Bürger's studies on the physiology of insulin

Following on his earlier observations of a biphasic blood sugar response in healthy individuals following physical exertion, Bürger, in 1920, had demonstrated a long-lasting hyperglycaemia on effort as a striking reaction in untreated diabetics (Fig. 1, A, B).

On the other hand, he found the hyperglycaemic effect of intravenous injections of dextrose to be moderated and shortened by muscular exercise. The occupational therapy,

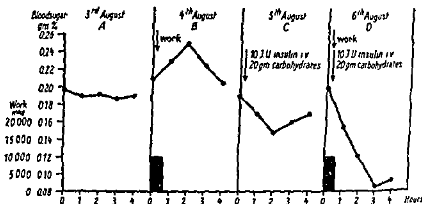


Fig. 1. Long-lasting hyperglycaemia on effort and enhancement of hypoglycaemic insulin action by additional work in a dietetically unregulated diabetic.

in diabetes, propagated by Bürger definitely potentiates the blood-sugar lowering effect of insulin, sometimes inducing a real hypoglycaemia, and thus exerts an insulin-sparing action (Fig. 1, C, D). Besides its practical consequences for the metabolic stabilization of diabetics in everyday life, this finding was the reason for Bürger's making further efforts to clear up theoretically the proper site of action of insulin in the organism. At that time the theory was generally accepted that the accumulation of glycogen was the principal function of insulin. Bürger regarded that idea as erroneous, for after intravenous injection of commercial preparations of insulin

Separation of glucagon from insulin

In 1930, Abel and Best were kind enough to supply Bürger with special preparations of crystalline insulin. This enabled him to explore the subject thoroughly under controlled conditions. By studying the effect of fourteen different preparations of insulin on fifty rabbits he established the following facts: (1) the intravenous injection of 0·1 mg. of dry insulin (any commercial specimen) produces an initial hyperglycaemia, varying in degree, within the first fifteen minutes; (2) on the other hand, the phenomenon is absent following the administration of crystalline insulins of the Abel and Best types, even when injected intraportally; (3) following treatment with boiling 0·02 N-sodium bicarbonate for thirty minutes, the hyperglycaemic effect is unaltered, whereas the blood-sugar lowering action of insulin is completely abolished; (4) the hyperglycaemic principle is found in the first muriatic fractions of alcoholic extracts from the pancreas, and is probably preformed in the pancreatic gland itself. It is regarded as an independent physiological substance acting as a synergist of insulin.

By these statements Bürger in 1931 had already outlined the [illegible] e inactiva- [illegible] suggested [illegible] For some years Bürger and his co-workers devoted themselves to this task.

The aim of his experiments was to show whether the hyperglycaemic principle (1) really represented a pancreatic hormone acting in the range of a few μg /kg. body weight, like the known action of insulin, or (2) was merely an impurity or a secondary degradation product of insulin; (3) whether it acted as a specific antagonist or as a physiological synergist of insulin, and (4) whether by any means it might be separated in a pure form from technical preparations of insulin.

The purification of the crude substance was, of course, the principal task. As a basic material Burger used a dry sample of Wellcome insulin; a dose of 20 μg./kg. of this caused, in

systematically, for similar action, all the commercial insulin preparations then available. He found the initial hyperglycaemia due to intravenous insulin injections to be a fairly constant phenomenon. The different preparations showed some differences in hyperglycaemic effect, the intensity and duration of which depended upon the actual dosage as well as on the mode of injection and on the glycogen content of the liver, respectively.

Following a diet rich in carbohydrates, and after intraportal injection of insulin, the effect was of particular intensity. On the other hand, it was absent in patients suffering from hepatic cirrhosis, jaundice and thyrotoxicosis, as well as after interruption of the liver circulation. The phenomenon was not absent after bilateral adrenalectomy in dogs nor after denervation of the hepatic hilum and intraportal insertion of a glass tube. Hence, it could not be due to a counter-regulatory release of adrenaline nor to a neurohepatic reflex mechanism. Furthermore, the interruption of muscular circulation did not influence it. Since, finally, continued chemical analyses of liver tissue proved a progressive glycogen depletion following intraportal injection of insulin, it was evident that only hepatic glycogenolysis could be the source of the initial hyperglycaemia.

At first, Bürger explained the "initial insulin hyperglycaemia" as *a paradoxical effect of the hormone itself.* The observation was confirmed in principle in numerous investigations by other writers later on. This original interpretation accounts for the difficulty of carrying out research work on insulin physiology at that time resulting from lack of pure preparations of insulin. Yet, from the beginning he was struck by those very *differences between the paradoxical* hyperglycaemic efficiency and the sometimes relatively low reducing power of commercial insulin preparations. This contrast led him to the question of whether the initial hyperglycaemia was not an effect of insulin itself, but rather was due to a concomitant physiological substance, or to an artifact of the special extraction procedure.

Separation of glucagon from insulin

In 1930, Abel and Best were kind enough to supply Bürger with special preparations of crystalline insulin. This enabled him to explore the subject thoroughly under controlled conditions. By studying the effect of fourteen different preparations of insulin on fifty rabbits he established the following facts: (1) the intravenous injection of 0·1 mg. of dry insulin (any commercial specimen) produces an initial hyperglycaemia, varying in degree, within the first fifteen minutes; (2) on the other hand, the phenomenon is absent following the administration of crystalline insulins of the Abel and Best types, even when injected intraportally; (3) following treatment with boiling 0·02 N-sodium bicarbonate for thirty minutes, the hyperglycaemic effect is unaltered, whereas the blood-sugar lowering action of insulin is completely abolished; (4) the hyperglycaemic principle is found in the first muriatic fractions of alcoholic extracts from the pancreas, and is probably preformed in the pancreatic gland itself. It is regarded as an independent physiological substance acting as a synergist of insulin.

By these statements Bürger in 1931 had already outlined the physiological significance of glucagon. The exclusive inactivation of insulin by boiling in weak alkaline solution suggested the possibility of separating glucagon from insulin. For some years Burger and his co-workers devoted themselves to this task.

The aim of his experiments was to show whether the hyperglycaemic principle (1) really represented a pancreatic hormone acting in the range of a few µg./kg. body weight, like the known action of insulin, or (2) was merely an impurity or a secondary degradation product of insulin; (3) whether it acted as a specific antagonist or as a physiological synergist of insulin, and (4) whether by any means it might be separated in a pure form from technical preparations of insulin.

The purification of the crude substance was, of course, the principal task. As a basic material Bürger used a dry sample of Wellcome insulin; a dose of 20 µg./kg. of this caused, in

rabbits, an initial hyperglycaemia of 18–24 per cent above the starting level. Inactivated and active insulin, as well as the *supposed concomitant substance, failed to pass the collodium* membrane. His attempts at purification by adsorption onto charcoal, aluminium oxide and Fuller's earth, and by chromatographic analysis using benzoic acid, were unsuccessful. At 38° the hyperglycaemic effect of Wellcome insulin could

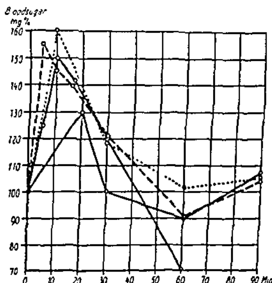


FIG. 8a. Hyperglycaemic effect of crude substance A_{4a} (glucagon) in a rabbit (dosage: 20 μg. given intravenously)

be enhanced markedly by treating for several hours with 93 per cent alcohol. By repeated fractionations and titration with pyridine, Bürger and Brandt finally, in 1935, obtained a highly effective substance from Wellcome insulin in an acetic acid solution, pH 4·38. This fraction was called A_{4a}, and a dose of 20 μg./kg. *of this caused, in rabbits, a 50 per cent rise* in the blood sugar level, lasting about one hour (Fig. 8*a*).

It was not yet entirely free from traces of insulin, as was evidenced by the abolition of the final hypoglycaemic phase

due to additional inactivation by alkaline solution (Fig. 8b). Nevertheless, Burger had obtained a substance so highly purified that its effectiveness surpassed that of most of the samples obtained later on by other workers. At an international meeting in 1931, Bürger noted the previous findings of Murlin, and from then on called the hyperglycaemic principle

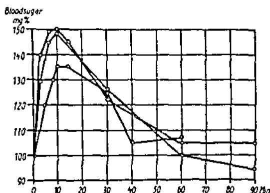


FIG 8b. Hyperglycaemic effect of substance A_{4a} (glucagon) in a rabbit (20 µg. given intravenously) after inactivation of contaminating traces of insulin by boiling in 0 02 N-bicarbonate solution

enriched in Fraction A_{4a} "glucagon", too. He computed its effective dose, active under physiological conditions, at least, as low as 5–10 µg./kg.

Chemical composition and identification of glucagon

Bürger's glucagon fraction proved insoluble in water, alcohol and other organic solvents, yet its salts were soluble in water as well as in dilute alcohol and acetone. The elementary analysis of the substance (Table I) revealed no marked differences from the values of various dry preparations of insulin with the exception of its somewhat lower sulphur content.

This finding is not so surprising, because the analytical results with substances of such high molecular weight will,

Table I

ELEMENTARY ANALYTIC VALUES OF GLUCAGON (FRACTION A_{44}) AND VARIOUS CRYSTALLINE INSULINS

	C per cent	*H* per cent	*N* per cent	*S* per cent	*Residue* per cent
Fraction A_{44} (Glucagon)	52 03	6 42	14·38	2·71	
Crystalline Insulin					
Abel	49 91	7·16	14 41	2·94	
Bonn, 'S' Bruch	48·64	7·03	14 02	3·08	0 49
Bonn, Stallmann	51·99	6·63	15·13	3·40	0·23

*evidently, be influenced by shifts of their elementary composi-*tion only if there exist great differences. In accordance with later electrophoretic results it may be concluded from the microanalyses that glucagon is rather similar to insulin in its physicochemical properties. Both must be proteohormones responding positively to Millon's and biuret tests and giving a colour reaction to 1·2 nitrosonaphthol.

The only outstanding feature which distinguished glucagon from insulin biochemically was that glucagon reduced potassium ferricyanide solution less readily than did insulin (Table II).

Table II

REDUCTION OF POTASSIUM FERRICYANIDE BY INSULIN AND GLUCAGON

	Amount of potassium ferricyanide reduced
1 g. crystalline insulin ('Best')	2·095 g
1 g. glucagon (Fraction A_{44}) .	1·287 g.

During inactivation by boiling in alkaline solution insulin splits off labile sulphur, whereas glucagon, which contains less sulphur, retains its activity in spite of this procedure; glucagon might therefore be considered as being only a cleavage-product of insulin. Obviously, this is not correct, as further hydrolytic treatment of inactivated insulin never yielded glucagon.

Meanwhile, Cori and Sutherland succeeded in separating glucagon from insulin electrophoretically. The migration speed of glucagon is slightly less than that of insulin, the isoelectric points lying close together. This may explain the difficulty in separating the two hormones chemically.

In 1953, Steigerwald and Schütte isolated glucagon by means of paper chromatography. In the same year, Staub, Sinn and Behrens isolated a crystalline substance from insulin which, in the very small dosage of 0·15 μg./kg. produced a marked hyperglycaemic reaction.

In spite of all this progress the chemical constitution of the hyperglycaemic factor, or glucagon, has not yet been clearly defined.

Action, biological identification and physiological importance of glucagon

From the investigations of Cori and Sutherland we have learned that with the exception of a few preparations the hyperglycaemic factor is still present even in most of the crystalline insulins. Bürger's missing the factor in the dry preparations tested by him has been explained by Weitzel as due to the fact that the zinc salts included in insulin crystals are attached to pyridine when subjected to Abel's original method of preparation. In 1953 Weitzel reported a correlation between the glucagon-like effect and the zinc content of crystalline insulins. The zinc salts as a complex organic compound may be extracted from the insulin crystals *in vitro* by means of transcrystallization in the presence of zincaffine acids and by suspension in analogous buffer solutions, respectively. If by those means one decreases the zinc content below 0·5 per cent, the glucagon effect of insulin can be abolished. The zinc protein compounds obtained by Weitzel from the buffer solutions were so effective that they raised the blood sugar markedly when administered intravenously to rabbits in a dose of 1 μg./kg.

Glucagon *is very active when administered intravenously*, but far less active when given subcutaneously It appears to

be destroyed by the enzymes of blood and tissues, like insulin, as was shown many years ago by Freudenberg, Karelitz and Bürger. It remains to be shown whether the intensity of this destroying power of the blood is related to the process of ageing, and whether it differs in healthy and diabetic individuals, as was established by Bürger with respect to insulin (Fig. 4).



FIG 4 Difference in the enzymatic insulin-destroying power of blood between diabetics and healthy individuals.

As Sutherland, Cori and others have demonstrated, glucagon exerts a direct influence on enzymatic glycogenolysis entailing conversion of glycogen to Cori-ester by activation of the hepatic phosphorylase system. This entirely confirmed Bürger's previous conception of an immediate glycogenolytic action of the hyperglycaemic principle on the liver cells. The hyperglycaemic efficacy of glucagon is, therefore, dependent on the glycogen content of the liver, as was shown by Bürger twenty years ago.

Lack of chemical micromethods for identification of glucagon in the blood and difficulties in the isolation of the pure substance in a stable form and in sufficient amounts added to the difficulty of studying the physiology of glucagon. Therefore, in the post-war years, Burger attempted to demonstrate the presence of insulin and glucagon in the bloodstream by indirect biological methods (Burger, 1947). His conception was based on the pattern of biphasic fluctuation of the blood sugar level after oral administration of dextrose. The final hypoglycaemic depression following sugar uptake is generally regarded as an effect of insulin. On the other hand, there is a difference of opinion on the mechanism of the initial hyperglycaemic phase. While this effect is partly attributed to a direct influx of sugar from the intestinal lymphatics, some authors are inclined to explain it in terms of a reflex mobilization of hepatic glycogen. Burger, following the latter theory, believed that dissimilation might be due to a reflex release of glucagon.

In order to investigate this problem he studied the effect of blood transfusion on the blood sugar regulation. In ten preliminary test series it was shown that a transfusion of about 400 ml. of blood taken from healthy donors before breakfast will not cause any marked alteration in the blood sugar level in healthy fasting recipients. In the subsequent main experiments the blood was taken from the donors after administration of 100 g. of dextrose, and in three different stages of alimentary hyperglycaemia. The transfusions produced typical reactions of the blood sugar level in the fasting recipients, these reactions evidently differing in course from each other within the different groups in a characteristic way.

The blood taken at the height of alimentary hyperglycaemia caused a considerable rise in blood sugar when transfused to the recipients. About thirty minutes after the transfusion the elevation reached its maximum of 35–48 mg. per cent above the initial value and lasted for about fifty-four minutes (Fig. 5).

If the venepuncture was performed at the beginning of the

alimentary hyperglycaemia of the donor the effect was a very slight one, and the average increase in blood sugar was 9 mg. per cent.

The recipient's response of an initial hyperglycaemia was entirely absent when the blood had been let in the declining stage of the alimentary hyperglycaemia of the donor. In this case, instead of a hyperglycaemic slope there resulted a

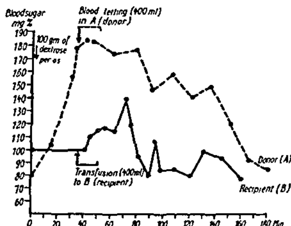


FIG. 5 Transfusion of a glucagon effect from donor (height of alimentary hyperglycaemia) to recipient.

marked decrease of blood sugar appearing as an insulin effect (Fig. 6).

Figure 7 shows the different types of reaction in relation to the venepuncture carried out at different stages of alimentary hyperglycaemia.

On comparison of these results with the different effects produced by infusion of 50–100 μg. of adrenaline, Burger is inclined to regard the transfusable hyperglycaemic reaction as being due to glucagon but not to an interaction of adrenaline.

Because of the contrasting influences of insulin and glucagon on the blood sugar level, some authors suggested that these hormones act as antagonists. Bürger disagrees with this from the physiological point of view and maintains that in general

insulin and glucagon should be regarded as synergists, and that in the mutual metabolic action of glucagon and insulin,

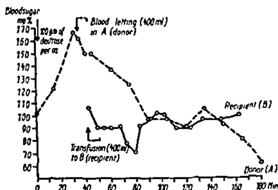


FIG. 6. Transfusion of an insulin effect from donor (declining stage of alimentary hyperglycaemia) to recipient.

the specific function of glucagon is to provide the peripheral muscles with sugar when necessary, by splitting off hepatic

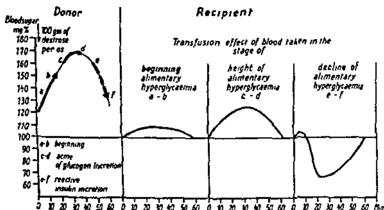


FIG 7. Biological demonstration of glucagon and insulin actions as proved by blood transfusions in different stages of alimentary hyperglycaemia

glycogen, the oxidation of which may then be helped by insulin. In any case, this conception of a successive release of

the two hormones is based on the phasic postponement of the blood sugar regulation as demonstrated by transfusion experiments.

Under physiological conditions the phasic separation of the reactions will probably not be so marked as might be deduced from those experiments. The reactive release of insulin will rather overlap the glucagon action and set in before the glucagon effect has expired. In addition to acute changes due to alimentary and physical stimuli the blood sugar level may be controlled by alternative release of the two hormones as a mutual trigger-mechanism depending on the actual blood sugar level and according to the glycogen supply in the liver and to the glucose demand of the muscle cells.

The final trend of the two pancreatic hormones is to accelerate the carbohydrate metabolism according to the requirements of the periphery, and for this purpose they are probably co-operative.

As was shown by Klotzbücher (1947, unpublished), in previous transfusion studies in Burger's laboratory, the blood of donors after physical exertion is apt to produce analogous reactions of blood sugar in resting healthy recipients. This might suggest that not only alimentary impulses but also physical effort is adequate to stimulate the release of glucagon.

Due to the methodological difficulties of an immediate determination it is not yet known how far adrenaline is in fact interfering with the mutual actions of the two pancreatic hormones. The influence of adrenaline on the regulation of blood sugar might be restricted to emergency reactions only, as *conceived by Cannon.*

Glucagon and diabetogenesis

Regarding pathogenesis and clinical aspects of diabetes mellitus, Bürger as early as 1935 outlined the potential significance of an excessive production of glucagon. He pointed out that probably *a continuous hypersecretion of that kind* might cause a deficiency of hepatic glycogen, and other

symptoms related to diabetes, even if the production of insulin were normal.

Some of the arguments in favour of this theory obtained by anatomical and experimental findings were described yesterday. I would like to add only that Klotzbucher (1953) reported glucagon-like reactions in healthy recipients after blood transfusions from diabetic donors.

Finally, most significant results were obtained by total pancreatectomy performed both under experimental and clinical conditions. We know that complete removal of the pancreas may improve the metabolic condition in alloxan diabetic rats. When carried out in human beings total pancreatectomy may involve an unexpected low substitutional need of insulin. The disturbance of the hormonal balance within the insulin-glucagon system, which by unilateral elimination of the insulin-producing cells gives rise to diabetes mellitus, may be compensated for by secondary or simultaneous removal of the tissue where glucagon is formed.

As far as I know, a permanent diabetes has not yet actually been produced by continued administration of glucagon. The facts discussed yesterday and mentioned before, however, might yield indirect arguments in favour of the existence of a "glucagon diabetes".

In some respects, the present stage of glucagon research resembles that of insulin during the years following the successful experiments of Banting and Best. There is clear evidence that glucagon is a physiological product of the pancreas, and experimental work yielded most important facts about the physiology and pathology of glucagon action. But technical difficulties have been delaying further progress in this field of research and have been hampering experimental studies on the pure substance under controlled conditions. Yet, we may be quite confident that when the technical difficulties are overcome the theoretical advance will involve practical consequences for the treatment of diabetes and other disturbances of the carbohydrate metabolism, and particularly the treatment of insuloma and Von Gierke's disease.

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DISCUSSION

Foà: Have you tried the transfusion of postprandial but not hyperglycaemic blood, such as blood obtained after a meal of fat, which would contain a number of assorted materials but no excessive amounts of glucose?

Schulze: No The experiments performed by Burger and Klotzbucher did not include investigations of transfusion effects of postprandial hyperlipemic blood They were restricted to the study of transfusion effects of blood samples taken in the [illegible]

[illegible] action is due to adrenaline.

C. F. Cori. Dr. Schulze, did you say that when blood was taken from the donor after exercise and injected into the recipient, that this pro- [illegible]

squeezing the fist once. It might be that there is a reflex mechanism that acts in response to physical impressions.

Ferner: There is no evidence that one will be able to produce a diabetes with injections of glucagon, but I think that in the physiological process glucagon is secreted neither subcutaneously nor intravenously but intraportally. I think that it has not been proved that diabetes mellitus can be produced by intraportal injections of glucagon

Schulze: As far as I know, no one has been able to produce glucagon diabetes of that kind, neither by intraportal nor by intraperitoneal injection.

Ferner: The physiological output of glucagon is into the portal vein, and the hormone is very rapidly destroyed by enzymes. It has also been found that implantation of β-cell adenoma does not cause a decrease in blood sugar.

Schulze: I only mentioned that as far as I know no one has succeeded in obtaining glucagon diabetes by continued administration of glucagon, wherever it is injected.

Ferner: Has it been injected into the portal vein?

Schulze: No.

Ferner: It must be proved.

Schulze: Of course.

Young: But, Prof Ferner, insulin is secreted also into the portal vein but one gets perfectly good effects by injecting insulin peripherally.

Ferner But it is not so rapidly destroyed.

glucagon.

They produced

tzel on

hich I

d not concern the

es of simple amino

Weitzel incubated

In this way the

zinc salts were extracted from the insulin crystals as protein compounds.

extremely small effective dosage.

de Duve. Yes, but have you or has Dr. Weitzel any comment on the fact that the purest samples of glucagon contain no zinc?

Schulze. Until recently, I had no information about those data clearly obtained with the new crystalline preparations of glucagon.

glucagon from insulin during recrystallization. In this case Weitzel prepared some complexes of zinc with very simple amino acids and showed that these complexes were blood-sugar raising at very low dosages.

Schulze · Weitzel's experiments which I mentioned in my presentation were not based on synthetic complexes of zinc with simple amino acids; he used zinc-protein extracts from crystalline insulin as a starting material.

Best: I think I did send Dr. Burger samples of insulin on one or two occasions. This was made by Dr. D. A. Scott in my department. Dr. Scott introduced the use of zinc in the crystallization of insulin. I can

still get the same effect. Dr. J. Salter and Dr F Gerritzen have been studying for some time the failure of the blood sugar to show a greater change after huge doses of glucagon-free insulin than it does after small doses.

Goldner. We too were interested in Dr. Weitzel's work with organic zinc compounds. Our biochemist, Mr Saifer, prepared zinc glycine

effect, furthermore, zinc glycine failed to give a hyperglycaemic effect. He then wrote to us and essentially agreed with our report.

Foà · The words 'insulin antagonist' and 'insulin synergist' have been

[illegible]

Young: Dr. Drury's experiments were with muscle?

Foà: Drury's were with eviscerated rabbits; Pincus's with liver and muscle of depancreatized dogs.

Goldner: To complicate matters further there is the paper of Van [illegible]

[illegible]

CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF GLUCAGON

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THE purification and crystallization of glucagon have been reported by Staub, Sinn, and Behrens (1953). Various studies have been undertaken to secure evidence concerning the purity of the crystalline protein. Zone electrophoresis experiments provided information indicating that the crystals were of high purity (Staub, Sinn, and Behrens, 1955). Similarly, amino end-group analyses yielded evidence of only one terminal amino acid. Upon repeated recrystallization no increase in biological potency was observed, and the specific biological activity of the residual protein in the mother liquor was as great as that of the crystals.

Elementary analyses are typically those expected of a protein (Table I). The low sulphur content sharply differentiates glucagon from insulin. A minimum molecular weight

Table I

CHEMICAL ANALYSIS OF GLUCAGON

C	H	N	S
50 11%	6 43%	17 45%	0 77%

HIS	METH	LYS	ARG	TYR	TRY
3 7%	3 7%	5 3%	10·6%	9 8%	6·5%

AMINO N	AMIDE N
0 83%	1 40%

calculated from the sulphur content, or the histidine and methionine values, is about 4,200. Preliminary ultracentrifugal studies and determinations of partial specific volume performed by Dr. E. O. Davisson and Mr. H. W. Fisher provided support for this figure.

Amino end-group analysis according to Sanger's dinitrophenylation procedure (Sanger, 1945) yielded only one N-terminal residue, namely, di-dinitrophenyl-histidine. Experiments were also undertaken to determine the C-terminal amino acid(s). The Akabori hydrazinolysis method (Akabori, Ohno, and Narita, 1952) clearly led to the release of threonine (Staub, Sinn, and Behrens, 1954). In contrast, the application of the carboxypeptidase technique (Harris and Knight, 1952) led to the liberation of a number of amino acids, and at least two of these, valine and leucine, were released as rapidly as threonine and may have preceded it. These methods have not led to unequivocal identification of the C-terminal group.

Following the characterization of glucagon as a small protein of high purity and consisting of a single peptide chain, attention was directed toward the possibility of determining the amino acid sequence of this substance. The quantitative amino acid composition is being determined. Studies have also been undertaken to determine the peptide fragments that are obtained following hydrolysis with enzymes or with mild acid. Trypsin appears to split glucagon into five fragments. The resulting peptides have been dinitrophenylated and separated on silicic acid partition columns; also the free peptides were separated on Dowex-50 columns. A peptide with tyrosine as the N-terminal amino acid has been isolated in essentially pure form. Its structure has been determined to be: Tyr-(Leu-Asp)-Ser-Arg. The structure studies are continuing.

Following the discovery of methods suitable for the purification of glucagon, studies on biological characterization were undertaken. The presence of glucagon in some commercial preparations of insulin has been recognized for many years, and has led to speculation regarding its effect on both

the biological assay and the hypoglycaemic action of insulin. This problem was studied in mice, using solutions of glucagon-free insulin and adding crystalline glucagon in amounts varying from 1·5 to 100 per cent by weight. There was no effect on the mouse convulsion test even when the ratio of insulin to glucagon was 1 : 1 (Root, Ellis, and Staub, 1954). When the blood sugar response of mice treated in this way was determined, it was observed that the curves were different during the first 30 minutes. In the presence of 50 per cent by weight of glucagon the blood sugar did not fall as rapidly as when insulin alone was injected, but the level reached at 30 minutes was the same. Apparently the glucagon hyperglycaemia is so brief that it is without effect during the period between 30 and 90 minutes in which the convulsions occur.

Similar results were obtained in rabbits. Upon addition of 1 or 10 per cent of glucagon, the hyperglycaemic effect was noted during the first 30–60 minutes, but the maximum hypoglycaemic response of the insulin was still observed.

The glucagon content of a number of samples of crystalline insulin has been determined on a quantitative basis (Staub and Behrens, 1954). The method for measuring the biological activity is an adaptation of the procedure for observing the hyperglycaemic response in cats described by Olsen and Klein (1947). Interference in the bioassay by the hypoglycaemic action of insulin was avoided by incubating the samples with cysteine. The crystalline zinc insulin preparations were found to contain 0·3–0·5 per cent of the factor. In view of the lack of effect of much larger quantities of glucagon on the insulin assay in mice and rabbits, it appears unlikely that these small quantities could significantly influence the clinical hypoglycaemic response.

Additional studies were undertaken to determine whether chronic administration of glucagon to normal animals may have a diabetogenic or any other effect (Root, 1954). Rabbits and rats were treated with glucagon daily for 6 months. The rats were injected intraperitoneally and the rabbits intravenously once a day with a dose of amorphous glucagon

equivalent to 140 µg. of the crystalline preparation. There was no indication of a permanent alteration of blood sugar, and no histological alterations of the pancreatic islets or other tissues were observed. The only effect that was observed was an increase in liver glycogen concentration (Fig. 1). The increase

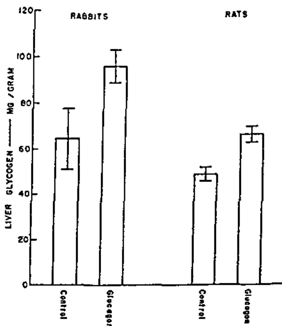


FIG 1. Effect of daily administration of glucagon (140 µg) for 6 months on liver glycogen of rabbits and rats.

was greater in rabbit liver than in rat liver but was statistically significant in both species.

Since glucagon stimulates increased glycogenolysis, it seemed likely that the increased liver glycogen stores in animals that had received repeated doses of glucagon might also lead to an increased hyperglycaemia response to a single dose of glucagon. Rabbits were injected subcutaneously twice daily with amorphous glucagon equivalent to 100 µg. of crystalline product. Control animals received saline injections.

Blood sugar responses to the morning injection were measured at various intervals. A typical response for one of the glucagon-treated rabbits is illustrated in Fig. 2. A striking increase in the hyperglycaemia is readily apparent.

Additional studies in rats were undertaken to investigate further this increase in liver glycogen concentration. To elim-

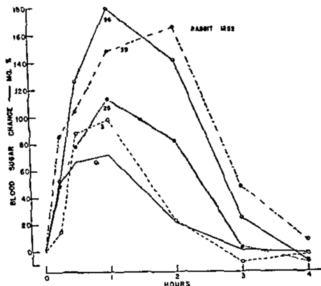


FIG 2. Blood sugar response to administration of 100 μg of glucagon.

The rabbit received 100 μg of glucagon twice daily. The number under each curve indicates the day of the experiment on which the blood sugar response to the morning dose was determined.

inate variations in food consumption as a factor in liver glycogen concentration, rats were force-fed twice daily with the medium carbohydrate diet described by Ingle (1953). The animals received twice daily a subcutaneous dose of amorphous glucagon equivalent to 100 μg. of the crystalline substance. The results of the experiment are shown in Fig. 3. Within 8 days the liver glycogen concentration was increased

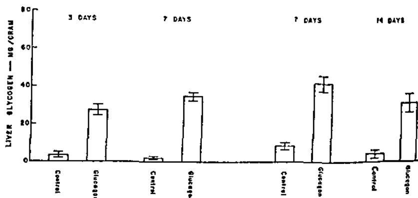


FIG 3. Liver glycogen of force-fed rats after twice daily subcutaneous administration of 100 μg. of glucagon.

Column height represents mean value; bars indicate standard error of the mean.

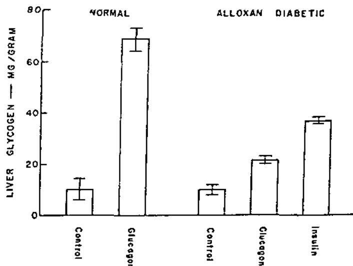


FIG. 4 Liver glycogen of force-fed rats after twice daily subcutaneous administration of 100 μg. of glucagon or after daily injection of PZI for 3 weeks.

Column height represents mean value; bars indicate standard error of the mean.

several fold; the effect was still greater at 7 days, but did not continue to increase at 14 days. The number of rats in each group was small, usually 5 saline controls and 10 glucagon-treated animals.

In a similar experiment alloxan diabetic rats were treated with glucagon or with insulin, and the liver glycogen was determined after 3 weeks. The response of the diabetic animals to glucagon was definite (Fig. 4), but not as great

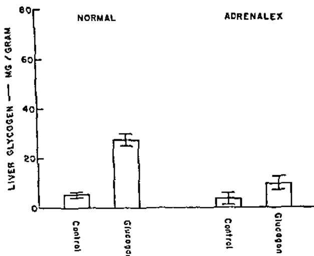


FIG. 5 Liver glycogen of force-fed rats after twice daily subcutaneous administration of 100 μg of glucagon for 7 days Column height represents mean value; bars indicate standard error of the mean

as in normal animals. The diabetes in the glucagon-treated animals was not changed; urinary excretion of sugar was similar to the control group. Liver glycogen of the insulin-treated animals was increased; however, in these animals the urinary excretion of sugar was decreased markedly, and the animals gained weight.

Experiments were undertaken to ascertain whether the adrenal gland played any rôle in the increase in liver glycogen produced by glucagon. Earlier work has shown that ACTH

caused an increase in liver weight and total liver glycogen but did not increase glycogen concentration (Helmer and Root, 1954). In contrast, glucagon caused no increase in liver weight, but did increase glycogen concentration. Adrenalectomized rats were treated twice daily with glucagon for 1 week. When they were killed for glycogen determinations, animals showing residual adrenal tissue were discarded. The glucagon-treated animals showed a significant increase in liver glycogen although not as great as that of non-operated animals receiving similar treatment (Fig. 5). The results suggest that the adrenals are not essential for the action of glucagon in promoting liver glycogen storage although they may be a factor in the large increases seen in normal animals.

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DISCUSSION

that bear on the point.

Foà: We have just concluded a series of experiments (Galansino, G., Weinstein, H. R., Magill, A. M., and Foà, P. P. (1955), *Amer. J. Physiol.*, **180**, 27) similar to those presented by Dr. Behrens and which might

N.Y. 85, 507) and ours, we found (Costa, M., Galansino, G., and Foà,

of insulin secretion through hyperglycaemia.

von Holt: We were interested in the effect of repeated glucagon

treated to destroy insulin?

Behrens: Our highly purified glucagon is substantially devoid of insulin. It has not been treated with alkali or cysteine.

Young: What are your criteria of freedom from insulin?

Behrens: We have depended upon the mouse convulsion test and upon blood sugar determinations in mice to detect residual insulin. In addition we have tried to detect the presence of cystine, an amino acid that

attributed to insulin in the glucagon, then the contamination would be of the order of 8 per cent. These observations confirm those made by R. H. Smith in our laboratory some years ago. I would not be prepared to say there is that much insulin there. We have some evidence that glucagon may enhance the action of insulin when both are added together *in vitro*, so that the rat diaphragm does not necessarily provide a true estimate of the amount of insulin present in samples of glucagon

Behrens: This might then indicate an upper limit?

Randle: Yes.

Foà: I should add that our glucagon was inactivated by alkaline incubation.

Best: We should remember that some of the earlier Lilly samples of

rule out a content of a few tenths of one per cent of insulin.

particularly since glucagon does not appear to increase the glycosuria of alloxan diabetic rats? Has glucagon been studied in depancreatized animals?

Behrens. We have not done it.

Foà: According to our experience the hyperglycaemic effect of single glucagon injections is greater in the depancreatized than in the normal dog.

R.-Candela: I can only refer to my early experiments. I studied the insulin requirement of alloxan diabetic dogs. After that I tied down the ducts of the pancreas and two months later we removed the pancreas. Within 24 hours the insulin requirement of the dogs decreased.

Best. I suppose care was taken to ensure that the amount of food absorbed was the same.

R.-Candela Yes. We fed the animals with raw pancreas and bread

We made a careful comparison of the insulin requirement of these

R.-Candela: No, only pancreatectomy.

Randle: Have the effects of duct ligation actually been carried out on insulin requirements of alloxan diabetic animals?

R.-Candela: Yes, first in a normal animal, then after alloxan diabetes

safe.

Best: I have seen diabetes result from ligation of the pancreatic ducts, after many weeks.

de Duve: With regard to the antagonism of insulin by glucagon, I think it is necessary to take into account the type of glucagon preparation used and the way it is administered. Certainly, when injected in-

which was described by Sutherland and Cori some years ago and according to present criteria should be about 10 per cent pure at the most. That preparation affected the blood sugar curve by insulin during 5 hours, instead of 30 minutes to 1 hour as described by Dr Behrens

data indicated that it is destroyed overnight. In the liver it is destroyed extremely rapidly. When we inhibit the destruction in the *in vitro* assay,

wonder whether we have any further information on the amounts of glucagon necessary, when given constantly intravenously, to prevent

per hour or of 170 μg. adrenaline per hour.

THE ACTION OF GLUCAGON ON LIVER PHOSPHORYLASE

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THE concentration of phosphorylase in liver slices represents a balance between inactivation and resynthesis of the active form (Sutherland, 1950, 1951, 1952; Sutherland and Cori, 1951). Inactivation of phosphorylase is catalysed by an enzyme earlier designated liver phosphorylase inactivating enzyme. The synthetic aspect of the balance, i.e. the reactivation of the inactive liver phosphorylase, is influenced by glucagon (and epinephrine) so that resynthesis of the active form is promoted.

The change from the inactive form to the active form was very rapid and the amount of active enzyme in slices was doubled within a few minutes following the addition of glucagon or epinephrine. In earlier studies the reactivation process could be demonstrated only in intact tissue; although once reactivation within the slice had occurred, the increased amounts of active enzyme could be demonstrated in homogenates, extracts, or in fractions of extracts obtained by ammonium sulphate precipitation. In order to study the nature of the changes in the phosphorylase molecule, it seemed advisable to purify the liver phosphorylase and the enzyme which inactivated it, since the inactivation reaction did occur in homogenates and could be studied in cell extracts.*

* The results summarized in this report have been submitted previously in detail to the *J. biol. Chem.*

I. E. W Sutherland and W. D. Wosilait (1956).
II. W. D. Wosilait and E. W. Sutherland (1956)
III. T. W. Rall, E. W. Sutherland, and W. D. Wosilait (1956).

Enzymatic Inactivation of Liver Phosphorylase

Enzymes used in these studies were prepared from dog liver. It was shown that the response of dog liver slices to addition of the hormones was similar to that seen when rabbit liver slices were used. Fig. 1 shows the effect on the concentration

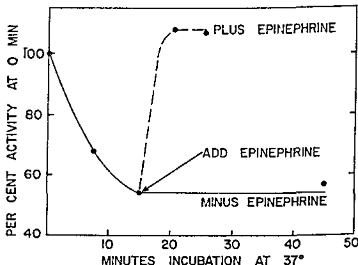


FIG. 1. Effect of
in dog liver slices.
30 ml. of medium
phosphate buffer (
indicated times and LP activity was determined (Rall, Sutherland and Wosilait, 1956).

of phosphorylase of incubating dog liver slices at 37°. Phosphorylase was measured in homogenates prepared from the slices. The concentration fell rapidly on incubation, reaching a low value in 15–20 minutes. Subsequent addition of glucagon or epinephrine resulted in an extremely rapid rise in the phosphorylase level. It should be noted that the dog liver phosphorylase had fallen appreciably during the slicing procedure; the true liver value was higher than the zero slice value given here.

Liver phosphorylase and the enzyme inactivating it were prepared in purified form from dog livers. Purified liver phosphorylase was inactivated enzymatically and a search was made for small fragments which might be released during the reaction. Inorganic phosphate was found to be a product of the reaction and was determined chemically by the method of Fiske and SubbaRow or by the Lowry-Lopez method. The enzymatic liberation of phosphate was determined not only by chemical measurements but by radioactivity when radioactive

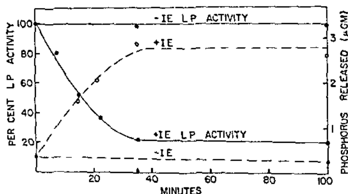
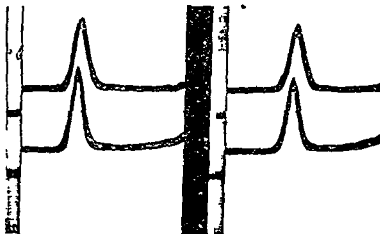


FIG. 2 Appearance of inorganic phosphate in the supernatant fluid of liver phosphorylase precipitated with trichloroacetic acid coincident with enzymatic inactivation (Sutherland and Wosilait, 1955)

liver phosphorylase was used as a substrate prepared as described in a later section. Formation of inorganic phosphate paralleled the formation of inactive liver phosphorylase as shown in Fig. 2 (Sutherland and Wosilait, 1955; Wosilait, 1955). The phosphate present in the molecule of liver phosphorylase was very firmly attached to the protein moiety and remained with the protein on precipitation with trichloroacetic acid; after enzymatic inactivation, the phosphate appeared in trichloroacetic acid supernatants. In this experiment 1 mole of inorganic phosphate was formed per 120,000 grams of protein, or approximately 2 moles of phosphate per mole of enzyme. No production of pentose

compounds, phenolic type compounds, peptides or ultraviolet absorbing materials was detected. Furthermore, no proteolytic activity was observed when the inactivating enzyme was incubated with several proteins. The inactive form of the enzyme sedimented in the analytical ultracentrifuge at the same rate as the active form; in either case only a single peak was noted, as shown in Fig. 3.

No phosphatase activity was noted when inactivating enzyme was incubated with a number of simple organic



phosphorus-containing compounds or with some phosphoproteins (Table I), but small amounts of phosphate were liberated from certain other phosphoproteins, namely phosvitin and α-casein. The activity of the enzyme on phosvitin and on liver phosphorylase paralleled one another when the enzyme preparation was subjected to various treatments as shown in Table II. Both activities were inhibited by 0·01 M-NaF and, for example, 0·15 M-NaCl, and neither activity was stimulated by magnesium ions. The activities were

Table I

POSSIBLY SUBSTRATES INCUBATED WITH IE

P released	*No P released*
Liver phosphorylase α-Casein Phosvitin	*Proteins* Casein β-Casein Ovalbumin *Non-proteins* α-Glycerophosphate p-Nitrophenyl phosphate 5-AMP ADP ATP Creatine phosphate *Phosphoenolpyruvate* Glucose-1-phosphate Fructose-1:6-diphosphate Ribose-5-phosphate

Table II

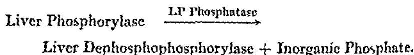
COMPARISON OF IE ACTIVITY ON LP AND PHOSVITIN

A comparison was made of effects on phosphate liberation from phosvitin with effects on enzymatic inactivation of LP. The extent of inactivation had been correlated earlier with the amount of phosphate released from LP.

Treatment	*Effect on activity toward.*	
	LP	*Phosvitin*
1. 0·01 M-NaF in R.M.*	95% inhibition	70% inhibition
2. 0 15 M-NaCl in R.M.*	78% inhibition	78% inhibition
3. 0 002 M-$MgCl_2$ in R.M.*	No effect	No effect
4. Pretreated for 15 minutes at pH 4 0 and 37°	Complete inactivation	Complete inactivation
5. Pretreated for 15 minutes at pH 8 0 and 50°	60–85% recovery	60–85% recovery

* Reaction mixture is described in text.

parallel following heat treatment; i.e. at pH 4 for 15 minutes at 37° all the activity was lost, while at pH 8 for 15 minutes at 50° most of the activity was retained. Only a small percentage of the total phosphate of phosvitin was released on long incubation, so it was not clear that phosphoserine proteins were the necessary substrates. The organic phosphate portion of liver phosphorylase is currently under investigation. The inactivation reaction at present may be written in the following form:


Liver Phosphorylase $\xrightarrow{\text{LP Phosphatase}}$

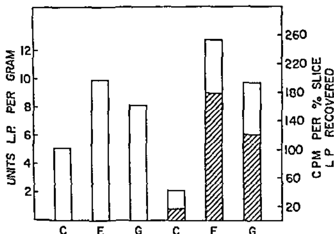
Liver Dephosphophosphorylase + Inorganic Phosphate.

Effects of Glucagon and Epinephrine on Incorporation of Phosphate into Liver Slice Phosphorylase

Since inorganic phosphate was released during enzymatic inactivation of phosphorylase, it seemed probable that the conversion of dephosphophosphorylase to phosphorylase consisted of a phosphorylation of the inactive form. Liver slices were known to incorporate inorganic phosphate rapidly into various esters (which might be capable of donating phosphate to the acceptor group of the inactive form) and on this basis experiments were designed to see if slices were able to incorporate phosphate into the inactive enzyme during the process of activation.

The following experiment, summarized in Fig. 4, demonstrated that ^{32}P-orthophosphate was incorporated rapidly into phosphorylase and that the amount of labelled phosphate present in the slice phosphorylase was much greater when slices were incubated with glucagon or epinephrine. Dog liver slices were preincubated for 15 minutes with buffer containing ^{32}P-orthophosphate to permit inactivation of phosphorylase and also entry of the labelled phosphate, then were further incubated for 5 minutes without and with the addition of glucagon (G) or epinephrine (E). At the end of the incubation,

the medium was decanted and the slices were homogenized in the presence of carrier liver phosphorylase. The bars at the left indicate the level of phosphorylase activity in the slices before carrier addition, and the bars at the right indicate radioactivity of the purified phosphorylase prepared from the homogenates. Purified samples from homogenates E and G



contained five to six times as many counts as the control sample. The cross-hatched portion of the bars represents the portion of the radioactivity that became TCA-soluble after enzymatic inactivation of the samples The phosphate present in liver phosphorylase did not exchange with substrate phosphate as the enzyme acted catalytically; this was shown by a series of experiments which will not be detailed here.

Comparison of Glucagon and Epinephrine with an Inhibitor of Inactivating Enzyme

With the additional knowledge and techniques now available, it was possible to study whether glucagon and epinephrine exerted their action by stimulation of the kinase

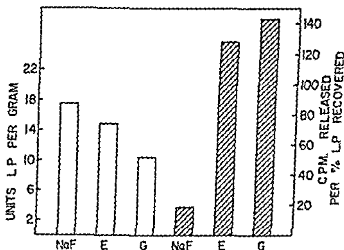


FIG. 5. Comparison of the effects of NaF, epinephrine and [illegible]

Sutherland and Wosilait, 1956)

system responsible for the donation of phosphate to dephosphophosphorylase in slices.

The incorporation of radioactive phosphate into phosphorylase during incubation with glucagon or epinephrine was compared to incorporation in the presence of fluoride, a known inhibitor of the inactivating enzyme whose addition to slices also results in an increased level of phosphorylase. Results of one such experiment are summarized in Fig. 5. The bars to the left represent phosphorylase activity of the slices at the

end of the experiment; the bars to the right represent the radioactivity of phosphorylase purified from the slices as determined by release of phosphate on enzymatic inactivation. Fluoride maintained phosphorylase concentration at a high level during incubation, and during this time very little radioactive phosphate entered the phosphorylase protein. Epinephrine and glucagon likewise maintained the level moderately well, but in these cases the radioactive phosphate was incorporated readily into the phosphorylase protein. This indicated that fluoride inhibited the inactivating enzyme, thereby preventing the removal of phosphate from the existing phosphorylase; consequently the added radioactive phosphate could not be incorporated in phosphorylase. Glucagon and epinephrine, on the other hand, acted by a different mechanism which indicated the effects resulted from a stimulation of the phosphate donor system.

Subsequent experiments supported this interpretation (Sutherland, Wosilait and Rall, 1955), as shown by an example summarized in Fig. 6. In this case the same experimental procedure was employed with only slight variation. In one flask (3rd bar) the slices were incubated with NaCl for 10 minutes, then both NaF and radioactive phosphate were added simultaneously. At this time the concentration of active phosphorylase in the slices had fallen to a low level. Addition of fluoride for the final 7·5 minutes increased the concentration of active phosphorylase very substantially, and during this period radioactive phosphate was readily incorporated into phosphorylase protein, as can be seen from the third cross-hatched bar. It was concluded that dephosphophosphorylase had been formed during incubation with NaCl and the addition of fluoride resulted in reformation of active enzyme, since the inactivating system was now inhibited and limiting, thus permitting the kinase action to become predominant. As this net formation of active enzyme proceeded in the presence of fluoride, radioactive phosphate was incorporated. Thus it appeared that the presence of dephosphophosphorylase was required for the incorporation of radioactive

phosphate into phosphorylase. The fourth pair of bars indicate that when the inactivating enzyme was inhibited by fluoride, the incorporation of phosphate was inhibited even in the presence of epinephrine, since only small amounts of dephosphophosphorylase had been formed.

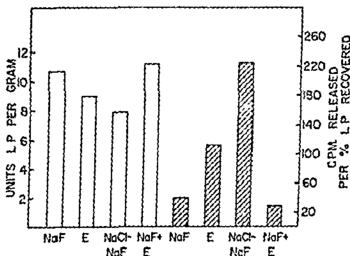


FIG. 6. Effect of adding ^{32}P and NaF simultaneously on incorporation of ^{32}P into liver slice phosphorylase. Four portions of slices were incubated 17.5 minutes under the conditions of Fig. 5. One mc. of ^{32}P was added to each portion 7.5 minutes before the end of incubation; at the same time the NaCl medium of one portion was decanted and replaced with NaF-containing medium. Cross-hatched bars at right refer to radioactivity made trichloroacetic acid soluble after enzymatic inactivation of final preparations.

Conversion of Dephosphophosphorylase to Phosphorylase with Soluble Kinase Preparations

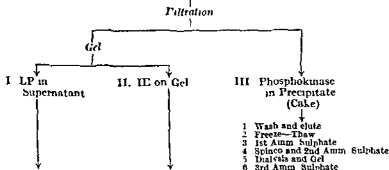
It was deduced from the experiments summarized above that a kinase was involved in the conversion of dephosphophosphorylase to phosphorylase. The protein-phosphate bond was firm and was not broken by precipitation with trichloroacetic acid nor by short incubation with hot dilute HCl or hot dilute NaOH, and the phosphate did not exchange

when incubated for prolonged periods with inorganic phosphate.

Studies on the reactivation process in broken cell preparations led to the finding of a phosphokinase* capable of transferring phosphate to dephosphophosphorylase. This enzyme was purified more than fifty-fold following the procedure summarized in Table III. Early fractionation steps

Table III

SCHEME FOR PURIFICATION OF LIVER PHOSPHOKINASE



separated the three enzymes rather well and permitted the preparation of the three enzymes from one homogenate. Dephosphophosphorylase kinase required adenosinetriphosphate and magnesium ions for the conversion of its substrate to the active enzyme. Requirement for ATP and Mg^{++} became evident simply by diluting the homogenate into a range suitable for assay.

The assay system which was adopted contained (as final concentrations) 1×10^{-3} M-ATP, $2 \cdot 5 \times 10^{-3}$ M-Mg^{++}, $2 \cdot 0 \times 10^{-2}$ M-NaF, buffer, and purified liver dephosphophosphorylase. From results in this assay system, it was possible to calculate that there was enough phosphokinase in liver to

* This enzyme has been named liver dephosphophosphorylase kinase, thus conforming to current terminology. For convenience phosphokinase is used as an abbreviation. It is pointed out that the use of the term kinase also conforms to an early definition where a kinase was a substance which transformed a zymogen to an enzyme.

activate all the phosphorylase in liver in a very short time—probably in one minute. This degree of activity is sufficient to implicate this enzyme in the rapid response of slice phosphorylase concentration to glucagon and epinephrine.

During the progress of this work, Fischer and Krebs (1955) reported the presence of an enzyme from rabbit muscle which catalysed the conversion of rabbit muscle phosphorylase *b* to phosphorylase *a* in the presence of ATP and manganous ions. A recent report from the Cori laboratory has described the PR enzyme action as a phosphorylase-rupturing action (Keller and Cori, 1955). It seems possible that the interconversions of phosphorylase in liver and muscle have some similar features, although the several enzymes involved differ physically or in other respects. The three enzymes discussed above have been purified from dog heart muscle to varying degrees in this laboratory. Highly purified inactivating enzyme from heart appears similar to the one from liver, and the very active phosphokinase from heart readily converts liver dephosphophosphorylase to the active form in the presence of ATP and magnesium ions.

Effects of glucagon and epinephrine on soluble phosphokinase have been obtained under certain conditions but have been variable. The work is too recent to present a clear picture, especially since cofactor requirements are present and inhibitory factors or processes also are operative in broken cell preparations. Results with slices, however, have supported the idea that some portion of the phosphokinase system is stimulated by glucagon and epinephrine.

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DISCUSSION

G. Cori: I should like to mention our knowledge of muscle phosphorylase as compared to liver phosphorylase. When Prof. Sutherland was in our laboratory he centrifuged his highly purified liver phosphorylase and found a sedimentation constant which was very close to muscle phosphorylase *c*, i e. the half-phosphorylase or monomer, whereas the crystalline phosphorylase *a* has twice the molecular weight. So liver phosphorylase either occurs only in this form or there is a species difference, because we have been working with rabbit muscle and Prof. Sutherland has used dog liver. Our PR enzyme, which Prof. Sutherland calls inactivating enzyme, does split phosphorylase *a*, the dimer, in half; whether inorganic phosphate is released in the process has not yet been determined. There is some phosphate released, but whether it is inorganic remains to be shown. It may be that the inactivating enzyme in liver and the PR enzyme in muscle are identical. The PR enzyme of muscle has been known to release organic phosphate from the dimer when it is split to two monomers, so it is quite possible that some of the contradictory or different findings can be brought under one heading. I would also like to say that we have tried our PR enzyme, which is highly purified, on α-casein. This enzyme is obtained from muscle which contains no monoestererases, whereas liver does, and our PR enzyme does not split phosphate from α-casein

Sutherland: We have not studied casein very much, phosvitin yes. Now if you apply this enzyme (IE) to whole casein it does not release

Young: What glucagon did you use in these experiments?

plex system so far.

C. F. Cori: I should like to say a few words about muscle, which we have chiefly studied. The systems of inactivation and reactivation of phosphorylase are extremely dynamic. In order to preserve the *status*

quo one has to freeze the muscle *in situ*, and then homogenize it in solutions that inactivate the enzymes that act on phosphorylase. If one does that one finds that within 10 seconds of stimulation at a rate of 20 per second the active form of phosphorylase increases. When muscle is fatigued the active form decreases markedly. A greatly increased glycogen breakdown takes place during contraction and it has always been a question how this could be brought about. It seems to be due to activation of phosphorylase, and that requires only 10 seconds. The purified system of Krebs converts inactive muscle phosphorylase to the active form. No effect has been demonstrated on this system *in vitro*, whereas it is well known that epinephrine does act on the intact muscle, presumably influencing the enzyme that converts the inactive to the active form. This effect disappears entirely when the cell structure is destroyed, it may be a question of the right experimental conditions, or there may be no hormone action on a cell-free, structureless system.

G. Cori: I have done quite a few experiments on frog muscle, incubating it with epinephrine or other substances which also increase lactic acid production and glycogen breakdown, such as caffeine, dinitrophenol; only epinephrine and norepinephrine increase the active form of phosphorylase Dinitrophenol and caffeine do increase glycogen breakdown to an even larger extent than epinephrine does. We find that the active form is not increased by these poisons. The amount of active form which is always present suffices to permit a considerable increase in lactic acid formation and glycogen breakdown. This breakdown is, of course, very much slower than the glycogen breakdown during stimula- [illegible] lot of active phosphorylase is needed to make possible [illegible] contraction.

[illegible] on the specificity of adrenaline derivatives and epin[illegible]?

G. Cori: I think Sutherland did some work on that which has been published Norepinephrine is certainly active, I do not know if it is as active as epinephrine, but it certainly is still active at 1 : 10 million, whereas, perhaps, epinephrine still acts at 1 : 50 to 1 : 100 million.

Sutherland We did titrate five or six different sympathomimetic amines by the liver slice technique. I think we published these results in 1949. In comparing the amines, if you take the half-maximal stimulation point and relate one to 100 per cent activity, you find that *l*-epinephrine is about six times more active than *l*-norepinephrine; the *d*-form of epinephrine has about the same activity as *l*-norepinephrine; *d*-norepinephrine has considerably less activity, and benzedrine is inactive. If you compare these results with the results published by McChesney and co-workers there is very good agreement. They studied these effects in the intact animal, and right down the line the parallelism is almost exact (McChesney, E. W., McAuliff, J. P , and Blumberg, H. (1949), *Proc. Soc. exp. Biol., N.Y.*, 71, 220)

C. F. Cori In the case of muscle, an action of glucagon on the phosphorylase system has not been demonstrated, while epinephrine does act. Now that one can determine by appropriate techniques the actual concentration of the active and inactive form, one finds that

normal rat muscle contains about 30 per cent of the active form, the remainder being the inactive form of phosphorylase. When one injects epinephrine into normal intact rats one finds, a few minutes after injection, an almost complete conversion of the inactive to the active form, while the total enzyme content remains the same. Norepinephrine in the same test on the intact rat is very much less active than epinephrine. Glucagon does not influence this system, either because it does not get into muscle or perhaps because it does not act in muscle.

Sutherland: These results are in good agreement with the work that has been published on the diaphragm (1950, *Proc. Laurentian Hormone Conf., N.Y.*, 5, 441; 1951, *Ann. N.Y. Acad. Sci*, 54, 693). I might add that while recent developments have not solved the exact mechanism of action of epinephrine, interesting possibilities can now be explored. It is possible that in some tissues phosphorylase is not the only enzyme affected. The phosphate donor system or the dephos- [illegible]

fluoride causes a rapid reactivation of the enzyme and this, I believe you have shown, goes together with a corresponding uptake of ^{32}P. It seems to me that this experiment shows that mere inhibition of the inactivating enzyme is enough to allow the kinase step to become preponderant and that the turnover of phosphorylase must indeed be very [illegible]

are consistent with such an interpretation, for we usually find a higher phosphorylase level in fluoride-treated slices than in those exposed to glucagon.

Sutherland: We have made calculations of the specific activity of the phosphorylase ^{32}P in slices Perhaps our calculations are not exact and we have not published them. We determine the specific activity of the inorganic phosphate and the specific activity of the phosphate from phosphorylase We find about 100 per cent equilibration during short times of incubation (5 minutes) If half of the phosphorylase were protected from dephosphorylation, we might expect the incorporation to be slowed considerably The equilibration, however, may be extremely rapid and the point you make is a good one. Some effects of epinephrine were 5-minute effects, i e, only 5 minutes of incubation time with radioactive phosphate was allowed.

INHIBITORY EFFECT OF GLUCAGON ON THE INSULIN GLUCOSE UPTAKE OF THE ISOLATED DIAPHRAGM OF THE RAT

J. L. R.-Candela and R. R.-Candela
Institute of Metabolism and Nutrition, Madrid

Results obtained from previous investigations (Candela, 1953; Candela and Candela, 1954) carried out with pancreatic extracts, and a few others carried out with rather impure glucagon, have motivated these experiments. We were interested in attempting to confirm these results and to establish whether glucagon has an inhibitory effect on insulin *in vitro* as studied in the isolated muscle. This inhibitory effect has been confirmed in studies on liver and muscle by Pincus and co-workers (1955).

Method

The experiments reported here were carried out, using a slightly modified form of the method already published (Candela, 1953), with Lilly samples of insulin, lot 208-108-B50, and of glucagon, lot 208-108-B234. Constant doses of glucagon (500 μg.) and varying doses of insulin were used.

Results

The results obtained are summarized in Tables I-IV, and are illustrated by Figs. 1 and 2.

Table I

INSULIN GLUCOSE UPTAKE OF THE ISOLATED RAT DIAPHRAGM

Number of experiments	*Insulin I.U./100 mg diaphragm*	*Insulin effect*	
		Glucose uptake	*Standard deviation*
12	0 0099	0 052	±0 019
12	0·1010	0 106	±0·010
12	0 3002	0 128	±0 013
24	0 5001	0·208	±0 025
16	0·7062	0·249	±0 031
12	0 9137	0 313	±0 019
12	1 1055	0 325	±0·023
12	1 2968	0 357	±0 016

Table II

INFLUENCE OF GLUCAGON ON THE INSULIN GLUCOSE UPTAKE OF THE ISOLATED RAT DIAPHRAGM

Number of experiments	*Insulin I U./100 mg. diaphragm*	*Insulin effect*	
		Glucose uptake	*Standard deviation*
12	0 0100	0 042	±0 007
12	0·1000	−0 070	±0 009
12	0 3007	−0 109	±0 012
20	0 4971	−0 195	±0 030
12	0 6994	−0 121	±0 021
24	0 9076	−0 077	±0 017
28	1 1095	−0 028	±0 022
12	1·2818	0·028	±0 025

Table III

INSULIN GLYCOGEN SYNTHESIS OF THE ISOLATED RAT DIAPHRAGM

Number of experiments	*Insulin I.U./100 mg. diaphragm*	*Insulin effect*	
		Glucose uptake	*Standard deviation*
16	0·0009	0·0237	±0·0013
16	0·0994	0·0333	±0·0049
12	0·3017	0·0451	±0·0104
12	0·4983	0·0563	±0·0039
16	0·6916	0·0594	±0·0193
16	0·9013	0·0769	±0·0052
20	1·1077	0·0919	±0·0066
16	1·2888	0·1059	±0·0184

Table IV

INFLUENCE OF GLUCAGON ON THE INSULIN GLYCOGEN SYNTHESIS OF THE ISOLATED RAT DIAPHRAGM

Number of experiments	*Insulin I.U./100 mg. diaphragm*	*Insulin effect*	
		Glucose uptake	*Standard deviation*
12	0·0100	0·0816	±0·0025
12	0·0998	0·0630	±0·0051
12	0·3033	−0·0296	±0·0018
12	0·4966	−0·0664	±0·0026
16	0·7008	−0·1347	±0·0134
16	0·8936	−0·1040	±0·0107
16	1·1111	−0·0711	±0·0173
20	1·2873	−0·0509	±0·0170

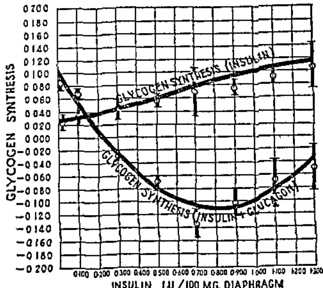


Fig. 1. Influence of glucagon on insulin *glucose uptake of the isolated rat diaphragm.*

Fig. 2. *Influence of glucagon on insulin glycogen* synthesis of the isolated rat diaphragm.

We conclude from the foregoing experiments and from their statistical interpretation that glucagon has an inhibitory effect on insulin *in vitro*, in the isolated diaphragm of the rat.

Figs. 1 and 2 show that the inhibitory effect of glucagon does not decrease proportionately to the increasing doses of insulin. If it did decrease proportionately, the maximum inhibitory action should be obtained with a minimum dose of insulin. This may explain why Young (1953) has not been able to demonstrate the inhibitory effect demonstrated above.

The continuous infusion of glucagon has been shown to inhibit insulin hypoglycaemia *in vivo* in the eviscerated and nephrectomized rabbit (Drury *et al.*, 1954). The administration of glucagon diminishes the transfer rate of glucose from the extracellular to the intracellular compartment. We have confirmed these results (Candela, 1955, unpublished).

These effects seem to be the specific property of glucagon, as a protein such as ACTH (Candela, 1955, unpublished) does not produce any effect whatever.

Acknowledgement

We should like to thank Dr. Kirtley and Dr. Peck of the Lilly Laboratories, Indianapolis, for gifts of insulin and glucagon.

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DISCUSSION

Young. What was the dose of glucagon used in the experiments shown in your figures?

R.-Candela: One mg.

Young: What was the effect of the glucagon alone?

R.-Candela: It diminishes slightly the glucose uptake in some cases.

In other cases there seems to be a slight increase in glucose uptake; there is no constant effect.

TABLE II (Randle)

Influence of glucagon upon the glucose uptake of rat diaphragm and its response to insulin.

Addition to buffer	*Concentration of hormone*		*Glucose uptake mg /g./hr. Mean ± S.E. of mean*
None	—	a	1 99 ± 0·14 (6)
Glucagon (1)	20 µg /ml.		3·97 ± 0·19 (6)
Insulin	6·4 mU./ml	b	3·48 ± 0 23 (6)
Glucagon (1) + Insulin	20 µg./ml. 6·4 mU./ml		4 37 ± 0·25 (6)
(b – a) difference not significant, other differences significant (minimum level of significance $P < 0\ 01$)			
None	—	a	1 44 ± 0·09 (5)
Glucagon (2)	180 µg./ml	b	2 38 ± 0·15 (6)
Insulin	1·8 mU./ml.		2 74 ± 0·13 (5)
Glucagon (2) + insulin	180 µg./ml. 1·8 mU./ml.		4·29 ± 0·28 (5)
(b – a) difference not significant, for all other differences $P < 0\ 001$			

Glucagon (1)—batch 208-158β-197 (Lilly)—amorphous
Glucagon (2)—batch 208-158β-220 (Lilly)—crystalline
Figure in parentheses is number of observations

Randle This slide (Table II) summarizes two experiments we have done on the influence of glucagon on the glucose uptake of rat diaphragm

the glucose uptake response to insulin. This figure for the glucose uptake —if one calculates the equivalent insulin activity of the mixture it would be compatible with the addition of the two effects of glucagon and insulin In the second experiment, using 180 µg /ml. glucagon, there was again significant stimulation of glucose uptake with glucagon, 1 8 milliunits

of insulin again produced significant stimulation of glucose uptake. When both hormones were present together, again there was no evidence of any inhibition by glucagon. If one calculates the equivalent insulin activity of this, then the recovery of insulin from this mixture is some

experiments confirm the earlier observations that were made in our laboratory by R. H. Smith and which were reported here by Prof. Young three years ago (Young, F. G. (1953), Ciba Foundation Coll Endocrin., 6, 241).

Young: It is true that in our laboratory we have never used such a high dose of glucagon as Prof. Candela has. What was the dose of insulin at the bottom of your curve?

R.-Candela: 0·1–1 unit.

Young: That is, 0·1–1 unit of insulin/0·5 mg. glucagon gives the maximum inhibition.

Randle: What incubation medium did you use?

R.-Candela: That used by Groen and Kaminga.

Randle: Did you dissolve your glucagon directly in the buffer?

R.-Candela: Yes.

Randle: In the experiments which we did it was dissolved in glycine buffer at pH 9·5 and then diluted with Gey's bicarbonate buffer.

Young: I think between R. H. Smith and yourself all sorts of methods have been used.

Randle: Yes.

Park: In a recent conference Dr. Wick pointed out that there is now

C. F. Cori: Other tissues, e.g. kidney, contain very little glycogen; adipose tissue does contain some. I was just wondering, if 20 μg. of glucagon stimulate the glucose uptake in the diaphragm—where is the specificity with respect to the effect of insulin? This is a bit disturbing to me.

two I wonder where we are.

Young: I think, Prof. Cori, you are inclined to doubt the specificity of insulin action on glucose uptake by the isolated diaphragm?

C. F. Cori: I do not doubt it, I just noticed that another protein in

amounts, presumably free of insulin, does have a fairly substantial

180 μg., and that was the crystalline one, and it was less effective. I have not done, and this will have to be done, a response curve for glucagon to see what relationship it bears to the response curve for insulin.

de Duve: I wonder if anyone has ever tried treating the glucagon with alkali or cysteine to inactivate whatever insulin might have remained.

Randle: I have not tried that and it must be done. Certainly the other

G. Cori: Do you think that crystalline preparations of albumin contain insulin?

glucose uptake of rat diaphragm which Dr.

impurity be detected?

Randle. I have not tried a very large amount of insulin with a relatively small amount of glucagon.

All these considerations make the inhibition of insulin action

R.-Candela: I tried only with ACTH.

using now.

Sutherland: At a previous meeting we heard that glucagon might protect insulin from insulinase activity, so that addition of glucagon might potentiate any traces of insulin either bound or added in the preparation.

C. F. Cori: One will have to look for insulin until proved otherwise.

R.-Candela: What is the work of Pincus?

Foà: Essentially the work was as follows: depancreatized animals

liver?

Foà: I do not know

THE HEPATIC ACTION OF INSULIN

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The hepatic action of insulin has been for many years a subject of controversy amongst physiologists. While there is no doubt that the diabetic state is accompanied by profound disturbances of liver metabolism which can be largely corrected by insulin treatment, the numerous negative or paradoxical results obtained on normal animals and on isolated liver preparations have led many authors to conclude that the effects observed on diabetics are due essentially to indirect mechanisms. As shown in previous reviews (de Duve, 1945; Bouckaert and de Duve, 1947), much of the work performed prior to 1945 has been vitiated by inadequate control of the blood sugar level in the experimental animals and by the presence of glucagon in the samples of insulin used. Since then, several investigators have been able to demonstrate a direct action of insulin on the liver, and the reality of this action has gained wider acceptance. More recently, however, new doubts have been raised by several investigators, whereas additional evidence in favour of a hepatic action of insulin has been obtained in this laboratory. It seems appropriate, therefore, to re-examine the problem in a critical manner.

Studies on Hepatectomized and Eviscerated Animals

As shown by Bouckaert, De Nayer and Krekels (1929), insulinized animals can be kept at a normal blood sugar level by the constant intravenous infusion of an appropriate quantity of glucose. The amount of sugar necessary to achieve this result, termed the "compensation dose", is fairly reproducible within a given species, but varies from one species to

another. It is determined empirically, small variations in blood sugar level above or below the normal value being corrected for mathematically (de Duve and Bouckaert, 1944). With this technique, the compensation dose was measured over a period of 80 minutes on normal fasted dogs and on hepatectomized or eviscerated animals receiving a supramaximal amount of insulin (de Duve, De Nayer, Van Oostveldt and Bouckaert, 1945*a*; de Duve, De Nayer, De Keyser and Bouckaert, 1945*b*). The main results obtained are summarized in Table I. From the considerable reduction in glucose requirement which follows removal of the liver, an organ which actually produces glucose in the fasting animal, it was concluded that insulin strongly promotes the hepatic uptake of glucose.

Table I

INFLUENCE OF HEPATECTOMY AND EVISCERATION ON GLUCOSE REQUIREMENT OF INSULINIZED DOGS

Results from de Duve and co-workers (1945*a*, 1945*b*).

Compensation dose: amount of glucose necessary to keep the animals at a blood sugar level of 87 mg. per cent during 80 min. following the intravenous injection of a large amount of insulin.

Animal	*No. of exp.*	*Compensation dose g./kg/hr. ± S.E.M.*
Intact dogs	7	1·57 ± 0·08
Intact dogs anaesthetized and ventilated artificially	8	0·87 ± 0·11
Hepatectomized dogs	6	0 30 ± 0 03
Eviscerated dogs	4	0·30 ± 0 05

Recently, these experiments have been repeated under slightly different conditions in two separate laboratories. Lundsgaard (1954) has compared the glucose requirement of intact with that of hepatectomized or eviscerated cats, given a large dose of insulin, and has found no difference. His values for the liverless preparations are comparable with ours and

the main difference lies in the compensation dose of the intact animals. These results, therefore, appear to indicate that the hepatic action of insulin is of much smaller importance in the cat than in the dog. It should, however, be pointed out that the intact animals were anaesthetized in Lundsgaard's experiment. This factor may decrease the compensation dose to quite an extent (Table I), probably by selectively affecting the hepatic uptake of glucose (de Duve *et al.*, 1945*b*). Also, since no increase in glucose requirement was observed following hepatectomy, the possibility that insulin inhibited the hepatic output of glucose in the intact cat still remains to be considered.

Lang, Goldstein and Levine (1954) have determined the peripheral uptake of glucose in intact and eviscerated dogs, by simultaneous measurement of blood-flow rates and of the arterio-venous blood glucose differences. Their values for the maximal uptake in the presence of insulin are 1·5 g./kg./hr. for the intact and 0·6 g./kg./hr. for the eviscerated preparations. The authors point out that these figures are very similar to the values of total glucose consumption obtained by the compensation technique (Table I) and conclude that the reduction in glucose requirement of the insulinized dog following hepatectomy is due, not to the removal of a site of active glucose metabolism, but to the suppression of a humoral influence exerted by the liver on the utilization of glucose by the muscles. In support of the existence of an influence of this sort, they cite cross-circulation experiments, in which the impaired peripheral glucose uptake of an eviscerated dog was raised to a normal value by a vascular connection with an intact animal.

The agreement between the values of Lang and co-workers (1954) and those of de Duve and co-workers (1945*a*) is deceptive, for the two groups of experiments were run at very different blood sugar levels. This is made clear by Fig. 1, in which both sets of data are plotted against the blood sugar level, as in the original papers. In order to accept the conclusion of Lang and co-workers (1954), one has to assume that

all the values obtained on intact animals by both groups of investigators define a single saturation curve, applicable to the peripheral as well as to the total glucose consumption, and reaching an identical maximum of 1·5 g./kg./hr. at a

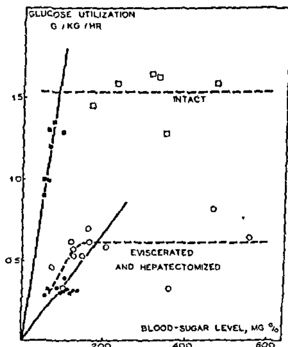


FIG. 1. Influence of hepatectomy or evisceration on utilization of glucose by insulinized dogs.

Dogs treated with a supramaximal amount of insulin and receiving a continuous intravenous infusion of glucose.

Black symbols: total utilization, results from de Duve and co-workers (1945*a*).

White symbols: utilization of hind-legs, results from Lang and co-workers (1954).

blood sugar level as low as 100 mg. per cent. Against this assumption may be quoted the results of Wierzuchowski (1936), who found that the glucose consumption of intact dogs becomes maximal only at very high levels of glycaemia and

may then reach 8 g./kg./hr., i.e. twice the value found at a normal blood sugar level. It must be pointed out also that in their experiments on non-insulinized animals, Lang and co-workers (1954) have found that the influence of the liver on the peripheral utilization of glucose becomes manifest only at blood sugar levels higher than 300 mg. per cent, a value far above that maintained in our animals. It may therefore be concluded from the present evidence that the interesting phenomenon brought to light by Lang and co-workers (1954) does not necessarily account for the difference in total glucose consumption of intact and liverless dogs, injected with insulin and kept at a normal blood sugar level.

Studies on Hepatic Glycogen Formation in Whole Animals

Glucagon and hypoglycaemia have been rendered responsible for the fall in liver glycogen observed by many authors to follow the injection of insulin (de Duve, Hers and Bouckaert, 1946). Consequently, the influence of insulin on the formation of liver glycogen has been reinvestigated in Prof. Bouckaert's laboratory, using glucagon-free insulin and a compensation technique whereby the animals were protected against hypoglycaemia. The results of these experiments, some of which have been published in condensed form (Legrand *et al.*, 1948; Hoet and Tyberghein, 1951), are summarized in Table II. They indicate that treatment of fasting rabbits with insulin, together with sufficient glucose to maintain an approximately normal glycaemia for a period of 3 hours, causes a significant increase in the glycogen content of the liver as well as of the muscles. It has also been shown that insulin protects rabbits against the fall of liver glycogen induced by adrenaline or glucagon (Tyberghein, 1952). Similar results had already been obtained with adrenaline by Cori, Cori and Buchwald (1930).

In some of the experiments described in Table II the mean blood sugar level of the insulinized animals was varied by

Table II

INFLUENCE OF INSULIN ON LIVER AND MUSCLE GLYCOGEN CONTENT

C: Controls: I: Animals injected with 30 units of glucagon-free insulin and receiving a continuous intravenous infusion of glucose during 3 hr.; *BSL: blood sugar level.*

Rabbits	*No. of animals*		*Mean BSL mg.%*		*Liver glycogen g. %*		*Muscle glycogen g. %*		*Reference*
	C	*I*	*C*	*I*	*C*	*I*	*C*	*I*	
Fasted 24 hr., anaesthetized with chloralose	12	14	94	107	0·79 ± 0·10	1 80 ± 0·19	0 54 ± 0·06	0·62 ± 0·04	1
Fasted 48 hr., anaesthetized	6	6	113	125	0·61 ± 0·11	1·84 ± 0·22	0·44 ± 0 05	0·62 ± 0·07	1
Fasted 24 hr.	12	12	104	116	1 14 ± 0·16	2·45 ± 0·08	0·47 ± 0·05	0·62 ± 0·05	2
Fasted 24 hr.	12	12	111	126	1·51 ± 0 30	3·62 ± 0·50	0·46 ± 0·05	0·59 ± 0·06	3
Fasted 24 hr., splanchnic denervated	12	12	107	105	3 42 ± 0·51	4·08 ± 0·54	0·49 ± 0·04	0·69 ± 0·03	3

1. Legrand *et al.* (1948); De Mayer, unpublished data.
2. Nys, unpublished data.
3. Hoet and *Tyberghein (1951).*

giving them amounts of glucose above or below the compensation dose.* As illustrated in Fig. 2, a significant correlation was observed between the quantity of glycogen deposited in the liver and the mean blood sugar level at which the animals

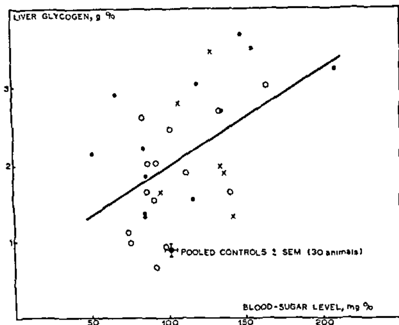


FIG. 2. Influence of blood sugar level on deposition of liver glycogen in insulinized rabbits.

Results from Legrand and co-workers (1948) and unpublished data of De Mayer and Nys (see Table II).

O : Animals fasted 24 hours, anaesthetized with chloralose
× : Animals fasted 48 hours, anaesthetized with chloralose
● : Animals fasted 24 hours, unanaesthetized.

were maintained. From the calculated regression line it can be deduced that the administration of insulin and glucose caused a mean increase in hepatic glycogen of 1·09 g. per

* [illegible]

Table II

INFLUENCE OF INSULIN ON LIVER AND MUSCLE GLYCOGEN CONTENT

C: Controls: I: Animals injected with 30 units of glucagon-free insulin and receiving a continuous intravenous infusion of *glucose during 3 hr; BSL: blood sugar level*

Rabbits	*No. of animals*		*Mean BSL mg.%*		*Liver glycogen g. %*		*Muscle glycogen g. %*		*Reference*
	C	*I*	*C*	*I*	*C*	*I*	*C*	*I*	
Fasted 24 hr., anaesthetized with chloralose	12	14	94	103	0·79 ± 0·10	1·80 ± 0·19	0·54 ± 0·06	0·62 ± 0·04	*1*
Fasted 48 hr., anaesthetized	6	6	113	125	0·61 ± 0·11	1·84 ± 0·22	0·44 ± 0·05	0·62 ± 0·07	*1*
Fasted 24 hr.	12	12	104	116	1·14 ± 0·16	2·45 ± 0·08	0·47 ± 0·05	0·62 ± 0·05	*2*
Fasted 24 hr.	12	12	111	126	1·51 ± 0·30	3·62 ± 0·50	0·46 ± 0·05	0·59 ± 0·06	*3*
Fasted 24 hr., splanchnic denervated	12	12	107	105	3·42 ± 0·51	4·08 ± 0·54	0·49 ± 0·04	0·69 ± 0·03	*3*

1. Legrand *et al.* (1948); De Mayer, unpublished data.
2. Nys, unpublished data.
3. *Hoet* and Tyberghein (1951).

lactate or pyruvate formed in the muscles from circulating glucose. In order to investigate this possibility, Dr. Hers has recently conducted a compensation experiment, using [1-^{14}C] glucose. A fasting rabbit was first given a priming dose of carrier-free sugar, calculated to bring the specific activity of the glucose in the blood and extracellular fluid to a desired level, and then injected with 80 units insulin and sufficient labelled glucose to keep the blood glucose and radioactivity approximately constant. A control animal, receiving no insulin, was rendered comparable by the infusion of carrier-free glucose. The evolution of the blood picture in the two animals is illustrated in Fig. 3. At the end of one hour, both rabbits were killed; the glycogen was extracted from the liver and muscles and degraded by means of the technique described by Hers (1955).

As shown in Table III, both the liver and muscle glycogens obtained from the insulinized animal were considerably more radioactive than those of the control animal and contained most of the label in carbon atom 1, indicating that they were

Table III

ANALYSIS OF GLYCOGEN FROM RABBITS TREATED WITH [1-^{14}C] GLUCOSE

Unpublished data of Hers. For details of experiment, see Fig. 3.

	Control		*Insulinized*	
	Muscle	*Liver*	*Muscle*	*Liver*
Specific activity, c./min./mg. glycogen	0	3·5	4·5	73·3
Percentage distribution of ^{14}C				
C_1	—	74·5	98·5	84·5
C_2	—	—	—	} 4·3
C_3	—	—	—	
C_4	—	—	—	0·7
C_5	—	—	—	3·2
C_6	—	—	—	3·2
Recovery	—	—	—	95·9

cent in animals maintained at the same mean blood sugar level as that of the controls (102 mg. per cent) and that the administration of excess glucose leading to a glycaemic increase of 100 mg. per cent brought about an additional glycogen deposition of 1·26 g. per cent. In both cases, the total amount of glycogen stored in the liver corresponded

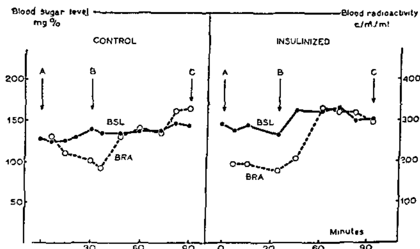


FIG 3. Evolution of blood glucose and blood radioactivity in rabbits treated with [1-^{14}C] glucose.

Unpublished data of Hers.
Experiment performed on 3 kg rabbits, fasted 24 hours.
Control: A. Priming dose of carrier-free glucose, 270,000 c /min.
B. Continuous infusion of carrier-free glucose, 270,000 c /min./hr
C. Killed
Insulinized: A. Priming dose of carrier-free glucose, 270,000 c./min
B. 30 units of insulin + continuous infusion of labelled glucose, 2 7 g /hr. and 810,000 c /min /hr
C Killed.

to about 15–20 per cent of the administered glucose. Roughly the same proportion was converted into muscle glycogen.

The evidence described above, although arguing strongly in favour of the existence of a direct action of insulin on liver tissue, is not, however, entirely demonstrative, since the newly formed hepatic glycogen could have originated, by a process independent of insulin, from intermediates such as

reported in a preliminary communication (Berthet *et al.*, 1954), a reproducible activation by added insulin of the synthesis of radioactive glycogen from uniformly labelled glucose was observed. These experiments have now been extended and stimulations ranging between 12 and 140 per

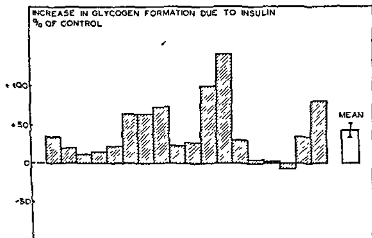


FIG. 4. Influence of insulin on liver glycogen formation *in vitro*
Results from Berthet and co-workers (1954), Berthet and Jacques (1955, unpublished).
Measurements are of radioactive glycogen formed by liver slices from fed rabbits, incubated 30 *min.* at 37° *in medium B of Hastings and co-workers* (1952) containing uniformly labelled [^{14}C] glucose. Insulin was added at the concentration of 1 Unit/ml.

cent have been observed in fifteen out of eighteen measurements (Fig. 4). On an average, insulin promoted the formation of labelled glycogen by 41 ± 9 per cent ($P < 0{\cdot}001$).

Other Metabolic Studies on Isolated Liver Slices

As first shown by Bloch and Kramer (1948), and later confirmed by Brady and Gurin (1950), Brady, Lukens and Gurin (1951), and Masri, Lyon and Chaikoff (1952), insulin added *in vitro* stimulates the incorporation of radioactive acetate into fatty acids by isolated rat liver slices. Similar observations have been made in this laboratory by Berthet and

formed directly from glucose, without a preliminary splitting of the molecule into smaller fragments. The same conclusion has been reached previously by Stetten and Klein (1946), from results obtained on animals given heavy water.

Studies on Hepatic Glycogen Formation *in vitro*

One of the strongest objections against the acceptance of a hepatic action of insulin has been the repeated failure to demonstrate an effect of the hormone on the formation of glycogen in isolated liver preparations. Three years ago, Hastings, Teng, Nesbett and Renold (1952) reported some preliminary observations indicating that insulin added *in vitro* promotes the incorporation of radioactive glucose into the glycogen of normal and diabetic rat liver slices. In a detailed account of these and other experiments, which has recently appeared, Renold, Hastings, Nesbett and Ashmore (1955) now state that they have been unable to reproduce this effect. Not only was added insulin inactive on liver slices from normal or diabetic rats, but it was necessary to treat the diabetic animals with insulin during a minimum of 24 hours in order to restore the capacity of the liver to convert glucose into glycogen, carbon dioxide and fatty acids. In contrast the metabolic defects of the isolated diaphragm from diabetic rats were repaired almost immediately following insulin treatment. In their discussion, the authors stress the scanty evidence supporting the existence of direct insulin effects on the liver and put forward the hypothesis that the slow hepatic changes brought by the hormone either are the result of a special type of action, different from the immediate activation of glucose uptake which insulin exerts on muscle tissue, or represent adaptation to changes in substrate concentrations resulting from the changes in peripheral metabolism.

In this laboratory, similar experiments were performed on liver slices from fed rabbits, using the incubation conditions first recommended by Hastings and co-workers (1952) for the demonstration of an insulin effect on rat liver. As has been

opinions of the authors. An attempt to investigate this problem experimentally has recently been made by Berthet and Jacques (1955). Rabbit liver slices were incubated in the

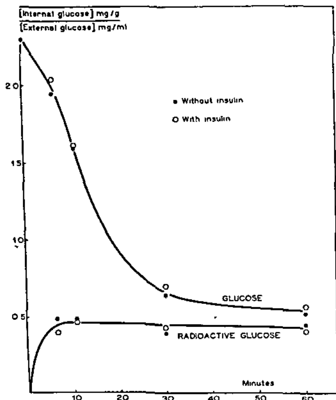


FIG. 5. Glucose exchanges between isolated liver slices and surrounding medium.

presence of radioactive glucose, taken out after varying times of exposure, blotted and analysed for free glucose and radioactivity. The results of an experiment of this type, performed with and without insulin, are shown in Fig. 5. At the start,

Jacques (1955, unpublished), using liver slices from fed rabbits and labelled fructose as precursor. As shown by Masri and co-workers (1952), insulin is without effect on fatty acid synthesis from acetate in liver slices from rats fasted 18 hours, but stimulates this process if the slices are incubated in the presence of glucose, a strong indication that the effect is an indirect one, mediated by an increased uptake of glucose. Similarly, Krahl (1953) has observed a favourable effect of insulin on peptide synthesis in liver slices from diabetic rats, the effect being demonstrable only if glucose was present in the incubation medium. According to Bornstein (1953), insulin added to normal rat liver slices reverses the inhibition of peptide synthesis caused by a β-lipoprotein fraction extracted from diabetic plasma.

Liver slices from diabetic rats show a strong impairment in fatty acid synthesis from a variety of precursors, but in this case insulin is without effect, whether added alone, with glucose, or with another intermediary metabolite (Brady and Gurin, 1950; Brady *et al.*, 1951). As with glycogen formation, it is necessary to treat the animals with insulin during 1–3 days, in order to restore the lipogenic function fully (Chernick *et al.*, *1950*; Chernick and Chaikoff, *1950*, *1951*; Felts, Chaikoff and Osborn, 1951*a*, 1951*b*; Osborn, Chaikoff and Felts, 1951; Lyon, Masri and Chaikoff, 1952; Osborn, Felts and Chaikoff, 1953; Renold *et al.*, 1955). Krahl (1953) has also obtained some evidence of a delayed action of insulin on the restoration of the capacity for peptide synthesis in diabetic liver.

Permeability Studies

Many authors believe that insulin exerts its metabolic effects on muscle tissue by promoting the transfer of glucose across the cell membrane. Since the liver always contains glucose, often at a higher concentration than in the blood, it seems unlikely, *a priori*, that a similar mechanism could account for the hepatic action of the hormone, and this objection has in fact been raised either against the existence of such an action or against the permeability theory, depending on the

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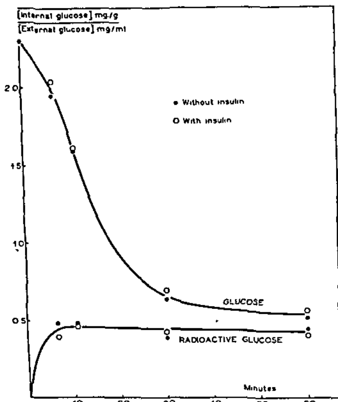


FIG. 5. Glucose exchanges between isolated liver slices and surrounding medium

Results from Berthet and Jacques (1955, unpublished)

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output of human subjects by simultaneous determination of blood-flow rates and arterio-venous blood glucose differences. They found that the intravenous injection of glucagon-free insulin results in an immediate depression of the glucose output of the liver, lasting approximately 30 minutes and followed later by an increased production of glucose, coincidental with the recovery from hypoglycaemia. In a number of cases negative output values were observed, indicating that the liver was actually taking up glucose from the bloodstream.

In normal subjects, the response was very constant, accounting for approximately 40 per cent of the total disappearance of glucose induced by insulin. Diabetic patients could be classified in two groups. The hepatic-sensitive diabetics showed an exaggerated liver response to insulin, representing up to three times the upper normal limit. These patients had a juvenile unstable type of diabetes. The hepatic-insensitive diabetics, who responded badly to insulin, were mostly elderly overweight patients, with a more stable type of diabetes and a tendency towards fatty change of the liver. Subjects of the hepatic-sensitive group were found to become hepatic-insensitive when severely ketotic.

Discussion

The response to insulin of the liver *in situ* has been estimated in three different ways: by the difference between intact and hepatectomized animals, by the increase in hepatic glycogen content, and by direct measurements of hepatic glucose output. The three approaches lead to the same conclusion, namely, that insulin increases the glucose uptake of the liver in the intact animal and that this process accounts for a significant part of the total glucose disappearance induced by the hormone. The relative importance of this effect, however, seems to vary from one species to another. It appears to be greatest in dogs and possibly humans, smallest in cats; rabbits occupying an intermediary position. Data on rats, unfortunately, are not available.

the slices are loaded with endogenous glucose. This leaks out but is partly replaced by further glycogenolysis, resulting in a fairly slow decrease of the internal glucose concentration, which approaches equilibrium with the external medium only after 30–60 minutes. At the same time, the external, radioactive glucose enters the slices rapidly, reaching equilibrium within less than 10 minutes. The two curves tend towards the same value, corresponding to a glucose space of the order of 0·5 ml./g.

At first sight, the results of Fig. 5 would seem to indicate that reversible exchanges of glucose between the liver cell and the external medium occur readily and are not affected by insulin. However, a space of 0·5 ml./g. represents distinctly less than the total free water of the tissue, and it appears therefore that part of the liver cell is not accessible to glucose. Consequently, the results obtained, though furnishing no positive evidence, show at least that some kind of transfer phenomenon could be involved in the hepatic action of insulin, provided the glucose-utilizing and insulin-sensitive systems of the liver cell are located in the glucose-free region. There are, indeed, indications that liver hexokinase, like the muscle enzyme, is not free to act in the intact tissue as it does in extracts, since both organs respond to changes in blood glucose concentration in a range far superior to the probable Michaelis constant of their hexokinases.* In both cases, the nature of the block remains to be established, a finding which would probably provide a definite clue as to the true mode of action of insulin.

Clinical Studies

Using hepatic vein catheterization, Bearn, Billing and Sherlock (1953) have been able to measure the hepatic glucose

us to distinguish between the hypotheses which have been proposed.

There remain, however, several facts which are not easily explained by the above theory and which indicate that the insulin-sensitive systems in the liver possess some peculiar properties, the significance of which is not yet understood. In the first place, there is a sharp contrast between the ease with which the action of insulin can be evidenced on isolated muscles and the great difficulties which have been experienced by most workers who have attempted to demonstrate a similar action on the isolated liver. Even where positive effects have been observed, their magnitude seems to be far below what could be expected from the results obtained *in vivo*. Also puzzling is the fact that insulin promotes fatty acid synthesis in rat liver slices, presumably by a mechanism involving an increased uptake of glucose, but that an increased glycogen formation could not be demonstrated on the same system. Most intriguing of all are the insulin-insensitivity of the diabetic liver *in vitro* and the slowness with which this organ regains its normal properties following insulin treatment.

In evaluating these discrepancies, it must be kept in mind that the liver occupies a unique position in metabolic regulations, being equally important in effecting the storage of foodstuffs as glycogen, fat and protein during the absorptive period, as it is in making body reserves again available to the tissues in the form of glucose and ketone bodies, in the post-absorptive state. How these antagonistic functions are prevented from competing with each other and allowed to become alternatively dominant is still a matter for speculation. Undoubtedly, hormonal effects play an important part in these mechanisms, but it seems reasonable to assume that the liver cell itself possesses some self-regulatory properties, depending on its morphological and functional organization. Of possible significance in this respect is the fact that glucose-6-phosphatase, a key enzyme in the production of glucose, is attached to microsomal particles (Hers *et al.*, 1949) which

These results were obtained with glucagon-free insulin. Undoubtedly, they would have been very different if the hormonal preparations used had contained the hyperglycaemic factor. The new data also serve to emphasize the importance of the blood sugar level. The danger of hypoglycaemia as a stimulus for the discharge of glycogenolytic agents has been stressed repeatedly and is again illustrated in the work of Bearn and co-workers (1953), who found the hepatic glucose output of the liver to increase as the result of hypoglycaemia. Independently of this indirect mechanism, there exists a direct relationship, almost of straight proportionality, between the glucose uptake of the liver and the blood sugar level. First demonstrated by Cori and Cori (1929), the sensitivity of the liver to fluctuations in blood glucose concentration has been well evidenced by Soskin and co-workers (1938). It is illustrated here by the steepness of the curve relating the glucose consumption of the intact animal to the blood sugar level (Fig. 1) and by the results on liver glycogen (Fig. 2). The increased liver reaction to insulin observed by Bearn and co-workers (1953) in the hepatic-sensitive diabetics could also be explained by the existence of hyperglycaemia in these patients.

Both by its promptness and by its nature, the response of the liver to insulin resembles that of the peripheral tissues and may be similarly explained on the basis of an accelerated conversion of glucose into hexose-phosphate, with the difference that, with the liver, one is dealing with an organ which spontaneously produces glucose. It is therefore reasonable to suppose that the observed effects are the result of a direct action of the hormone on the hepatic tissue itself and not of some indirect process, involving other endocrine glands or the nervous system. Supporting this conclusion are the data summarized above, showing that effects of added insulin can, under certain conditions, be demonstrated on isolated liver slices from normal animals. As already pointed out, the basic mechanisms involved in the action of insulin could be similar in liver and muscle tissue, but the available data do not allow

glucokinase activity in the liver requires the presence, at frequent intervals, of high concentrations of glucose in the portal blood. These observations afford some support to the hypothesis that liver glucokinase may have the character of an inducible enzyme and further suggest that the glucose formed in the liver does not have the same effect on the formation of the enzyme as the glucose entering the organ by way of the portal vein. This point is of interest with respect to the possible compartmentation of the liver cell, referred to above. It should, however, be noted that insulin did not prevent the impairment of glucose tolerance observed by Hill and co-workers (1954) following fructose feeding.

Acknowledgement

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have been shown to be débris of a delicate intracellular network forming the endoplasmic reticulum (Palade and Siekevitz, 1955).

If the assumption is made that some kind of intracellular compartmentation is necessary for a normal hepatic reaction to insulin, it becomes conceivable that small structural alterations brought about by suppressing innervation and a normal blood supply, isolating the tissue and exposing it to an unnatural medium, suffice to lower considerably its capacity to take up glucose and to react normally to insulin. In addition, the influence of metabolic changes must also be taken into account, in view of the results of Bearn and co-workers (1953), who showed that the liver of ketotic patients becomes resistant to insulin.

Possibly, these interfering alterations occur more readily in the liver isolated from untreated diabetic animals. That such may be the case is suggested by the contrast which exists between the slow recovery following insulin treatment of the diabetic liver, when tested *in vitro*, and the experimental observation that in many instances diabetic animals or patients exhibit a fairly rapid and normal liver response to injected insulin. Other factors which may have to be considered are possible liver damage caused by alloxan and the existence of hormonal imbalances, since properties characteristic of the diabetic liver may be induced, *in vivo* and, in some cases, *in vitro*, by means of growth hormone, cortisone, adrenaline or glucagon (Brady *et al.*, 1951; Haugaard and Stadie, 1953; Haugaard and Haugaard, 1954; Berthet *et al.*, 1954; Berthet and Jacques, 1955, unpublished). Finally, the possibility, considered by several authors, that insulin deprivation causes the loss of one or more essential enzymes, is one of great interest, deserving further study. Hill Baker and Chaikoff (1954) have recently shown on rats that the replacement of glucose by fructose in the diet during as little as three days causes a decrease of the glucose tolerance, apparently related to an impaired utilization of glucose by the liver. The authors put forward the view that the maintenance of a normal

DISCUSSION

Best: We did some work on this timing of the appearance of glycogen after the administration of insulin and certainly within a few hours you can see the increase of glycogen.

G. Cori: We had the same result some years ago.

Young: Have you determined the position within the glycogen molecule of the radioactive glucose taken up either in the intact animal or in liver slices?

de Duve: Yes, we have in a way, although not in as elegant a way as

increased liver glycogen

G. Cori: I was very interested in Prof. de Duve's reference to this disturbing information about the enzyme glucose-6-phosphatase. Why does glucose-6-phosphatase not always act on its substrate, preventing it from being transformed to glycogen by way of glucose-1-

central vein, while the opposite is true for glycogen

Best: Dr. A. Rappaport in my laboratory has been working for some years on the liver lobule, and all his evidence suggests the view that the periphery of the lobule is the area near the central vein, from the physiological point of view. He has demonstrated a system of liver acini which he can outline very clearly. It is true that in anoxia you see the first signs of damage in the areas near the

achieved that dubious honour!

Kerly: I would like to ask Prof. de Duve if the slide showing the

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no detectable production of ketone bodies by the liver until after 2–3 days of fasting, in the dog.

Lawrence: It takes 36 hours of starvation of normal man to produce ketosis.

[illegible]

tive to detect physiological variations in the production of ketone bodies

Young: Many experiments of this sort can be criticized technically on the basis of lack of blood-flow measurements. It is very difficult to be certain in this matter.

Best: I think probably there is quite a species difference here again One would expect an earlier appearance of ketosis in the cat than in the dog. In support of Prof. de Duve's conception, there might be some increase in the rate of utilization of the ketone bodies when they are presented in larger amounts. This would delay the appearance of ketosis.

Young: Utilization in the liver?

[illegible] There are several [illegible] ing has quoted, [illegible] ketosis I am [illegible]

and co-workers (Deane H. W., Nesbett F B, Buchanan, J M, and Hastings, A. B (1947), *J. cell comp Physiol.*, 30, 255) have shown that in the best [illegible]

glucose-1-phosphate as substrate.

Sutherland: Inorganic phosphate accumulates in slices It appears very rapidly no matter how carefully you handle these slices. I have often wondered where that phosphate comes from I wonder whether anyone here has information on that.

C. F. Cori: It may come from phospholipids There are not enough organic acid-soluble phosphate esters present to produce as much inorganic phosphate as one gets during incubation of a liver homogenate.

increase, due to insulin, in the glycogen content of the liver slices was based on measurements of radioactivity or on estimations of glycogen content. Last year, after reading a report of Hastings and his co- [illegible]

have used, whereas we, and I think most other people investigating glycogen synthesis, had always used fasted animals. We repeated experiments in which insulin was added to the incubation medium, using slices from fed rats. In the first group of experiments we got an increased glycogen synthesis, that is the increase in glycogen content was [illegible] r been [illegible] erfect [illegible] I was [illegible], like [illegible]

3 weeks, but our impression was that this was excessive and perhaps 2 days would have been enough. I think it is possible that it takes the liver an appreciable time to alter its enzyme pattern.

de Duve: There are undoubtedly a number of points which remain to [illegible]

types of diet.

Lawrence: Clinically, the change produced by insulin as presumed in the diabetic liver from a state of fat catabolism with ketosis to freedom from that is very short, 4 hours.

Young: Prof. de Duve, you assume that during normal post-absorptive periods the liver is releasing ketone bodies into the circulation. Is that quite certain?

[illegible]

SOME PROBLEMS OF PERMEABILITY OF TISSUE CELLS TO SUGARS

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IT IS comparatively easy to deal experimentally with the permeability of single cell preparations. Erythrocytes have been studied extensively in the past, and a great deal is known about the kinetics of sugar uptake, about the selectivity of the erythrocyte membrane toward various sugars, and about mutual inhibition of the penetration of two different sugars (LeFevre, 1954). Other cells have been studied less extensively, but enough is known to arrive at the conclusion that one cannot safely transfer the results obtained with one type of cell preparation to another.

Ascites Tumour Cells

In the past two years a study has been undertaken of the characteristics of permeability of the Ehrlich ascites tumour cell. Of these largely unpublished experiments, carried out by Drs. Crane, Field and Heimberg, only a few results will be mentioned. Equilibration between sugar concentration outside and inside the cells is reached so rapidly at 37° that one cannot measure it, and even at 20° equilibration is approached within a few minutes. The rate of penetration is not widely different for utilizable and non-utilizable sugars, including various pentoses, and pairs of sugars show mutual inhibition of uptake (Crane, 1955). The tumour cells differ from those of the human erythrocyte, intestinal mucosa and kidney tubule where glucose penetrates very much faster than do the pentoses.

Schulze: Has any experimental work been done to elucidate the influence of ketone bodies on the activity of the hepatic phosphorylase system? From clinical aspects it is our impression that ketosis in human

ketosis and is thus impairing the metabolic situation.

C. F. Cori: Perhaps one should talk about ketosis, because one of the prominent effects of lack of insulin in the liver is the fact that fatty acids instead of being synthesized are being broken down One knows now that the same enzymes are required for fatty acid synthesis and breakdown, and it seems that a certain amount of glucose catabolism is necessary for fatty acids to be synthesized, and we may readily connect this with the level of reduced DPN.

Fatty acid synthesis may be an indicator for glucose uptake; the same has been shown by Krahl for peptide synthesis. We have secondary and tertiary effects of the lack of insulin and they need not be corrected immediately. They certainly need not be corrected in liver slices where the repair mechanisms are apt to be slow.

Randle: One has to remember, too, that the severely diabetic animal is not merely deficient in insulin. In man, in severe diabetes, there is evidence for hypersecretion of adrenal steroid S (McArthur, J. W., Sprague, R. G., and Mason, H. L. (1950), *J. clin. Endocrin.*, **10**, 307; Stowers, J. M. (1951), *Clin. Sci.*, **10**, 487). There is evidence also of inceased adrenocortical activity in the alloxan diabetic rat (Rose, S. (1951), *Aust. J. exp. Biol. med. Sci.*, **29**, 469).

cent plasma sugar, fall on this line, which means that if the extracellular space were 15 per cent of the fluid volume of muscle, no measurable amount of sugar could be detected within the muscle cell. As a general and rather important point it should be mentioned here that the sugar concentrations inside the cell which would have a strong effect on

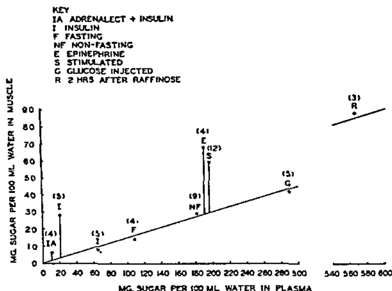


FIG. 1. Relationship between fermentable sugar in plasma and muscle.

the rate of the hexokinase reaction fall within a range which cannot be measured with accuracy by present methods. The K_m of hexokinase for glucose, determined by Crane and Sols (1954) for various tissues, is of the order of 1×10^{-5} M. A ten-times higher concentration inside the cell, which should give a nearly maximal rate of hexokinase activity, would correspond to only 2 mg. of sugar per 100 g. of tissue. It is

The uptake of sugars by the tumour cells as well as the mutual inhibition of sugar uptake of pairs of sugars was found to obey Michaelis-Menten kinetics; it should be emphasized, however, that this does not permit any conclusions to be drawn about the mechanism of sugar uptake, except to say that it is probably not a process of free diffusion. It was also found that the Michaelis constant for glucose was of the same order when determined with intact tumour cells or with hexokinase extracted from the cells, that is, K_m was close to 1×10^{-5} M in both cases. A barrier to the penetration of glucose should give a greater K_m value for the intact cells than for the isolated enzyme. Although the tumour cells have one of the highest rates of carbohydrate metabolism known for mammalian cells (equivalent to a glucose uptake of 40 mg. per g. wet weight per hour at 37°), the rate of penetration is obviously adjusted to the rate of metabolism. In fact, it would appear that in these cells the activity of the intracellular enzymes rather than the permeability through the cell membrane determines the rate of glucose metabolism Other single cell preparations are currently being tested in a similar manner.

Intact Animals

The question of the rate of penetration of sugar into the tissues of the intact animal is not so easily analysed. A study carried out previously on normal rats is pertinent in this respect (Cori, Closs and Cori, 1933). The fermentable sugar was determined in muscle and in plasma. In Fig. 1 the individual values previously published have been averaged (number of cases in parentheses) and recalculated in terms of concentration in the fluid volume of plasma and muscle, it being assumed that plasma contains 6 per cent and muscle 12 per cent solids. The straight line shown is a theoretical one for an equilibrium of plasma sugar concentration with 15 per cent of the fluid volume of muscle.

Referring to the key, it may be seen that four points, marked I, F, NF and G, covering a range of 60–290 mg. per

1951; Crane and Sols, 1954), and it is believed that this can cause an accumulation of free glucose inside the muscle cell. At least, this is the interpretation given to the experiments in Fig. 1, and other experiments, which will be reported in a later paper.

S in Fig. 1 refers to tetanic stimulation of muscle (three 10-second tetani), which caused a marked accumulation of free glucose inside the muscle cell. One of the reasons why these experiments were undertaken was the finding that heart muscle and diaphragm, two continuously active muscles, were much more permeable to glucose than resting skeletal muscle. In Table I, previously published values for heart

Table I

FERMENTABLE SUGAR IN HEART MUSCLE

Recalculated from published data (Cori, Closs and Cori, 1933) Figures in brackets refer to number of experiments averaged. Concentrations are given per 100 ml of water in plasma and muscle, respectively.

	Fermentable sugar (mg per 100 ml.)		
	Plasma	*Heart muscle*	*Intracellular**
Insulin (5)	68	26	12
Non-fasting (6)	161	66	34
Glucose (4)	303	195	158

* Calculated for an extracellular space of 25 per cent of heart muscle volume.

muscle have been recalculated in the same manner as in the experiments in Fig. 1. At a plasma glucose concentration of 160 mg. per cent, there was a measurable amount of free sugar inside the cell, even if one allowed for an extracellular space of 25 per cent of the fluid volume of heart muscle, and at higher plasma sugar levels the intracellular sugar concentration increased rapidly to about 50 per cent of equilibrium. This is in marked contrast to resting skeletal muscle, but can be imitated in skeletal muscle by subjecting it to stimulation.

impossible to determine with certainty whether such small amounts of glucose are inside the cell or not, because of the difficulty of measuring the extracellular space of muscle with such a degree of accuracy.

The point marked R in Fig. 1 refers to recent experiments in which raffinose (100 mg. per 100 g. rat) was injected intravenously into nephrectomized rats. Analyses were carried out 2 hours after injection. At this time an apparent equilibrium had been reached between sugar concentration in plasma and in tissue fluids, as judged by a steady level of raffinose in plasma between 2 and 4 hours after injection. It may be seen in Fig. 1 that raffinose distributed itself in a space corresponding to 15 per cent of the fluid volume of muscle, which is assumed to represent the extracellular space. In several of the experiments to be reported later raffinose was injected together with other sugars in order to have an independent measure of the extracellular space, based on the distribution of raffinose.*

It will be apparent that any point falling above the straight line in Fig. 1 represents an intracellular distribution of sugar. There were two conditions found which led to an accumulation of glucose *inside the muscle cell. These are marked* E and S, respectively. E refers to the subcutaneous injection of epinephrine, which has been shown to cause a marked increase in the hexosemonophosphate content of muscle. Severe insulin hypoglycaemia had a similar effect on hexosephosphate content, provided the adrenals were intact, which pointed to a secondary output of epinephrine (Cori and Cori, 1931). The accumulation of free glucose inside the muscle cell in severely hypoglycaemic animals disappeared when the adrenals were removed (see points marked I and IA in Fig. 1), and was therefore attributed to epinephrine secretion. Glucose-6-phosphate has since been shown to be a potent inhibitor of mammalian hexokinase (Weil-Malherbe and Bone,

* Note added in proof: Since this was written, additional experiments with raffinose have given a somewhat larger value for the extracellular space of muscle than that reported above, of the order of 20 per cent.

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In heart muscle, on the other hand, the rate of penetration was obviously greater than the rate of utilization, and intracellular glucose concentrations were reached which were far higher than the saturating concentration for hexokinase and hence of no significance for the rate of glucose utilization. Here again no answer is obtained beyond the fact that the rate of penetration is not rate-limiting for the glucose metabolism of heart muscle.

Penetration of Pentoses

The use of pentoses for the study of these problems offered a number of advantages. They are utilized at a slow rate and they can be determined by a specific method. We chose that of Roe and Rice (1948) rather than an orcinol method, because the former gives no blank in blood and tissue extracts deproteinized with barium and zinc and is not interfered with by other sugars, except when they are present in very large concentration. The pentoses used were D-xylose, D-lyxose, and L-arabinose, related configurationally to D-glucose, D-mannose and D-galactose respectively. In addition, experiments were carried out with D-arabinose related to D-altrose, and D-ribose related to D-allose. The kidneys were removed by a dorsal approach in animals anaesthetized with amytal. The sugars were injected into the femoral vein. At the termination of the experiments a gastrocnemius muscle was dissected free and cut at its insertion, and blood was drawn from the vena cava with a heparinized syringe.

In preliminary experiments the whole body of nephrectomized rats was extracted with hot water, deproteinized with [illegible]

cent for L-arabinose, 15 per cent for D-lyxose, 40 per cent for D-ribose and 20 per cent for D-xylose. D-Raffinose did not disappear at all, while glucose disappeared about twenty-five times more rapidly than xylose. Injection of insulin (1–1·5

In the experiments illustrated in Fig. 1, the rates of glucose utilization and hence of glucose uptake must have varied considerably between the fasted and the glucose-injected animal, but this is not reflected in the sugar concentration in muscle. The same point is illustrated in experiments on rat diaphragm shown in Table II. The hemidiaphragm with

Table II

Relationship of Glucose Concentration in Isolated Rat Diaphragm to Rate of Glucose Utilization

Data from Bornstein and Park (1953). The glucose concentration in the medium was 11 µM per ml. and the temperature of incubation 37°.

Time of incubation	*Glucose content of diaphragm*	*Glucose utilized by diaphragm*
minutes	µM per g	µM per g.
0	1 2	—
5	3 4	—
60	3 2	14
60	3 1	26*

* With insulin in medium

insulin in the medium utilized nearly twice as much glucose as the corresponding hemidiaphragm without insulin, without there being a significant change in the free sugar concentration in the diaphragm. One would be inclined to reason that facilitated transport across the cell membrane should increase the steady state of concentration of free sugar in the tissues, while increased activity of intracellular enzymes which utilize sugar should have the opposite effect. When this reasoning is applied to an actual experimental situation, e.g. the two examples just given, it can be seen that although one or the other mechanism may have been in operation, no decisive answer is obtained. This supports the contention previously made that the intracellular glucose concentrations which are of significance for the activity of hexokinase fall within a range which cannot be measured.

dehydrogenase reaction). The fall in the pentose level was partly due to increased utilization, and the column marked "calculated" refers to what the pentose level would have been if this had been the only effect of insulin. A comparison of the columns marked "calculated" and "found" shows that insulin has some additional effect, which if it were due to

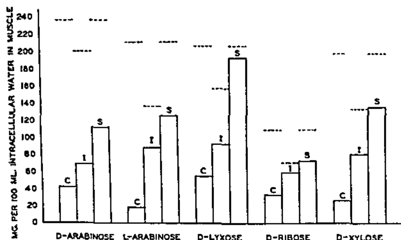


FIG. 2 The intracellular distribution of pentoses in muscle The extracellular space was assumed to be equal to the raffinose space (15 per cent of muscle volume) The amount of pentose present in the extracellular space was deducted from the total amount of pentose present in muscle in order to calculate the intracellular concentration per 100 ml of water. The dashed lines represent the concentration of pentose per 100 ml of water in plasma Values obtained 2 hours after injection

increased distribution of the pentoses would give the changes recorded in the last two columns.

The question was whether one could actually detect such an altered distribution by analysing various tissues. So far muscle has been analysed. In Fig. 2 are shown average values for the intracellular concentration of various pentoses in muscle 2 hours after injection. The dashed lines indicate the plasma sugar concentration, which if it were reached in the

units per 100 g.) increased the disappearance of all the pentoses except D-arabinose, and an even greater effect was achieved by stimulating both hind legs for 2 hours. It is as yet unknown what kind of enzymes act on these pentoses in mammalian tissues. In lower organisms various kinases have been found which phosphorylate pentoses in position 5 with ATP as phosphate donor. This position is available only in the furanose form of the pentoses, which is a form of low abundance in the mutarotation equilibrium mixture.

Table III

CONCENTRATION OF SUGARS IN PLASMA

Values obtained 2 hours after injection of 100 mg. of sugar per 100 g. nephrectomized rat and expressed in mg per 100 ml. of plasma water.

Sugar injected	*Concentration in plasma*			*Per cent distribution in body**	
	Control	*Insulin*		*Control*	*Insulin*
		Calc.	*Found*		
D-Arabinose	237	233	202	33	44
L-Arabinose	210	179	137	38	50
D-Lyxose	206	183	156	41	48
D-Ribose	107	83	70	54	61
D-Xylose	197	166	131	41	52
D-Raffinose	565	—	—	18	—

* Corrected for utilization of sugars.

In Table III are shown average values for plasma sugar [illegible]

per 100 g.) caused a decrease in the plasma level of the pentoses, in addition to a decrease in the glucose concentration (measured by means of the hexokinase-glucose-6-phosphate

Discussion

The transport mechanism for sugars, as far as it has been studied with various single cell preparations, seems to have the following characteristics. The effect of sugar concentration on the rate of penetration (or rate of exit) is described by a Michaelis-Menten type of equation rather than by an equation for a simple diffusion process. An apparent K_m (and K_i) can be evaluated for different sugars, and when two sugars are present they inhibit each other competitively in accordance with their affinity for a common transport process. In a diffusion process, e.g. through a cellophane membrane, one sugar would not inhibit the diffusion of another and the rate would not reach a maximum with increasing concentration. The Q_{10} for the transport process is between 2 and 3, i.e. the energy of activation is similar to that of an enzymatic reaction.

In intestine glucose, 3-methyl glucose and galactose are absorbed rapidly by an "active" process which requires energy, while the pentoses are absorbed slowly, apparently by a process of diffusion. Glucose and galactose show mutual inhibition of absorption (Cori, 1926), but it is not known (as far as the writers are aware) whether this is also true for a mixture of glucose and pentose.

In the experiments reported in this paper the following possibility had to be considered. If glucose were to inhibit the penetration of the pentoses into muscle, the insulin effect on the distribution of pentoses could have been due to a lowering of the extracellular glucose concentration, which undoubtedly took place as judged by a marked fall in the blood glucose level. In this case it should be possible to prevent the insulin effect on the distribution of pentoses by injecting enough glucose to prevent hypoglycaemia. It was found, however, that neither the insulin effect nor that produced by stimulation could be counteracted by the injection of large amounts of glucose or 3-methyl glucose.* This suggests

* Note added in proof: Similar results have since been obtained with galactose. The increased penetration of this sugar into muscle under the

intracellular water of muscle, would indicate an equal concentration of sugar inside and outside the cell.

Penetration of the pentoses into muscle is a slow process which is measured in hours, while penetration of glucose is measured in minutes. At comparable plasma sugar levels, D-lyxose and D-arabinose seemed to enter the muscle cell more rapidly than L-arabinose and D-xylose, but in no case was the equilibration greater than 30 per cent. On occasion a hematocrit determination was carried out and pentose determined in whole blood and plasma, from which the concentration in the erythrocytes could be calculated. The pentoses penetrated somewhat more rapidly into the erythrocytes than into muscle, but had not reached an equilibrium distribution 3 hours after injection.

Insulin increased the rate of penetration of the pentoses into muscle. An even greater effect was obtained by stimulating one gastrocnemius muscle through its nerve at a rate of 3 per second for 30–120 minutes, as compared to an unstimulated control muscle in the same animal. Once the pentose had entered the muscle, it remained there for long periods owing to the slow rate of utilization. Thus, 90 minutes of rest, after the injection, followed by 30 minutes of work gave a concentration of D-xylose of 120 mg. per 100 ml. of intracellular water, as compared to 38 mg. for the resting control muscle, at a plasma level of 198 mg. per cent. When the conditions were reversed, i.e. 30 minutes of work followed by 90 minutes of rest, the D-xylose concentration was 102 mg. per 100 ml. of intracellular water, at a plasma level of 196 mg. per cent. When one muscle was stimulated in an insulinized animal, it contained a higher intracellular pentose concentration than the resting control muscle. Since insulin lowered the plasma pentose level, the ratio of internal to external pentose concentration often reached unity in the stimulated muscle. These and other experiments will be reported in detail elsewhere.*

glucose (1×10^{6} to 1×10^{-4} M) fall within a range which cannot be determined analytically. On the basis of an analysis of the effect of stimulation and of insulin on the penetration of pentoses into muscle, an additional mechanism has been discussed, namely, an increased rate of diffusion through a membrane rendered more permeable by intracellular metabolic changes. As yet no decisive answer appears to have been obtained as to which of these mechanisms is in operation in cells which respond to the administration of insulin with an increased glucose utilization.

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[Discussion of this paper was postponed until after the paper by Dr. Park.—ED.]

either that muscle has a separate transport system for pentoses, or (what seems more likely in view of the slowness of penetration) that the pentoses enter muscle by diffusion through a relatively impermeable membrane. Moreover, the insulin effect is not specific for any particular configuration of the pentose molecule and is duplicated by stimulation of muscle.* Muscular activity is also known to increase the glucose consumption in diabetic muscle (Ingle *et al.,* 1951). There are thus a number of similarities between the effects of muscular work and of insulin on the carbohydrate metabolism of muscle.

It should be emphasized that the cell membrane is known to be affected by an altered metabolism within the cell. Thus muscular work might make the cell membrane more permeable to a number of substances which normally diffuse slowly into the muscle cell. These and other questions need further investigation, since in the opinion of the writers it has not been established with certainty how insulin produces its effect.

Summary

Two mechanisms have been proposed to explain the increased glucose consumption in skeletal muscle under the influence of insulin. In one case it is assumed that the activity of an intracellular enzyme (hexokinase) is increased, in the presence of a sufficiency of substrate, while in the other a specific transport mechanism is supposed to operate at an increased rate, providing more substrate to an as yet incompletely saturated hexokinase. It has been pointed out that in both cases the significant intracellular concentrations of

influence of insulin was not counteracted by the injection of large amounts of glucose, resulting in a glucose level in the blood 3 6 times higher than that of galactose.

respects the earlier procedures of Levine and co-workers (1950), Drury and Wick (1951), and Wick and Drury (1953). These modifications introduce the following advantages: (1) the hormone effect can be localized to the cells of specific tissues; (2) the effect on transport can be observed with metabolizable as well as non-metabolizable sugars; (3) the intracellular product of membrane transport can be determined; and (4) greater sensitivity is obtained.

In general outline, the procedure used was as follows. The sugar to be studied was infused intravenously into eviscerated-nephrectomized rats for a period of one to two hours. The concentration of sugar was then determined in the water of the blood serum, muscle and brain tissues. An effect of insulin on transport through the cell membrane could be ascertained from the change in ratio of the free sugar concentration in the tissue to the concentration in the blood serum. If the sugar remained in the extracellular water, ratios of 0·15–0·25 would be expected on the basis of the space available for distribution (Manery and Hastings, 1939; Nichols *et al.*, 1953; Nichols, 1954, personal communication). However, if the sugar entered the relatively large volume of intracellular water, these ratios would rise. Values as high as 1·0 might be reached with free passage of the substance into the cells.

Insulin effect on the transport of glucose analogues in muscle

Several hexoses and pentoses other than glucose were tested by this procedure. In Figs. 1 and 2 are shown the muscle to serum concentration ratios obtained for each sugar in the presence and absence of insulin. These ratios, shown by the height of the columns, should be compared to the broken lines across each pair of columns. These lines show similar determinations for mannitol, a substance which remains in the extracellular water and whose distribution is not affected by the hormone. In the absence of insulin the ratios for all sugars were the same or smaller than for mannitol, indicating

THE TRANSPORT OF GLUCOSE AND OTHER SUGARS ACROSS CELL MEMBRANES AND THE EFFECT OF INSULIN*

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THIS paper reviews our recent studies concerned with action of insulin. Two subjects in particular will be presented: (1) the action of insulin on the transport of glucose and other sugars into the cells of muscle and brain, and (2) the mechanism of transport in the cell membrane of the erythrocyte. No attempt will be made here to review the background of these topics. Discussions pertinent to the present work on insulin will be found in the papers of Cori (1945, 1949), Krahl (1951), Park (1952), Levine and Goldstein (1952), and particularly in the comprehensive review of Stadie (1954). Discussions relevant to glucose transport will be found in Davson and Danielli (1952), Ponder (1948), Ross (1951, 1952), LeFevre (1954), and Widdas (1952, 1954). Preliminary reports of the work reviewed here have appeared earlier (Park, 1953, 1954, 1955; Park and Johnson, 1953; Morgan *et al.*, 1955) and will be presented with more detail in forthcoming papers (Park *et al.*, 1955; Park and Johnson, 1955).

The Effect of Insulin on the Membrane Transport of Glucose and Other Sugars in Muscle and Brain

General description of the experimental procedure

In order to determine the effect of insulin on the transport of sugars across cell membranes, we have modified in several

* This work was supported in part by grants from the National Science Foundation, U.S. Public Health Service, and Eli Lilly and Company.

and diaphragm. These factors promote diffusion and thus make more sugar available at the cell surface for transport through the membrane. It should also be noted that the ratios varied somewhat with the sugar studied,* being somewhat lower in the case of ribose.

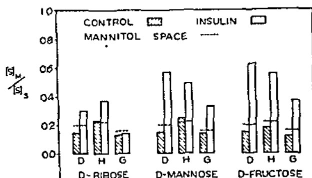


Fig 2. The effect of insulin on the distribution of D-ribose, D-mannose and D-fructose in the muscles of eviscerated rats (for abbreviations and procedure, see Fig. 1).

In each pair of columns the ratio with insulin is significantly different from the control ($P < 0.1$ per cent), except for xylose in the gastrocnemius.

Effect of insulin on glucose transport in muscle

The experiments shown in Fig. 3 are concerned with the transport of glucose, the sugar of principal physiological interest. The procedure was as described, except that the infusion period was only one hour in some experiments, as indicated on the chart. In the absence of insulin the ratios of the tissue to serum glucose concentrations, $[G]_M/[G]_S$, indicated that the sugar had an extracellular distribution. In the presence of the hormone, however, free sugar was present in large amounts in the intracellular water of the diaphragm and heart. In both the control and insulinized series these distributions were not influenced by the level of the blood sugar over the range of 50–1000 mg. per cent (Park and

* We have recently obtained data showing that insulin also promotes the entrance of the methyl pentose, L-fucose.

an extracellular distribution. In the presence of insulin the ratios were increased well beyond the limits of an extracellular distribution. Since these sugars do not arise by intracellular reactions, insulin must have accelerated their transport into

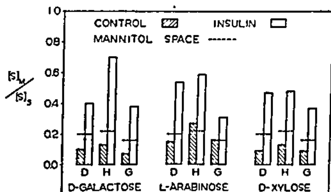


FIG 1. The effect of insulin on the distribution of D-galactose L-arabinose and D-xylose in the muscles of eviscerated rats.

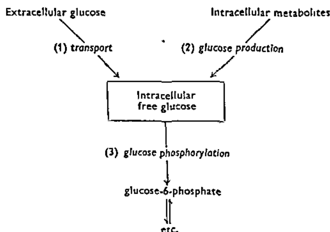
Abbreviations $[S]_M/[S]_S$, the ratio of the concentrations of

the cell from the interstitial water. It can be seen that insulin caused a higher distribution in the diaphragm and heart than in the gastrocnemius. This difference is probably due to the greater blood supply and the mechanical motion of the heart

insulin in intact rats (Fig. 3, left panel). In the control animals the ratios were determined only at normal blood glucose concentrations, since higher levels might cause insulin secretion. The control ratios indicated an extracellular distribution of the sugar. As seen previously, with insulin administration higher ratios were observed in the diaphragm and heart, indicating the presence of free intracellular glucose.

In the experiments shown at the extreme right of Fig. 3, glucose was infused into alloxan diabetic rats. The ratios indicated little or no intracellular free glucose in the absence of insulin. With administration of the hormone, intracellular glucose appeared in the diaphragm and heart, although to a lesser extent than in the eviscerated series. No effect was observed in the gastrocnemius.

We have interpreted the action of insulin on glucose as follows. The free glucose content of the muscle cell will be a function of three processes, as illustrated by the following diagram:



A rise in glucose content could be the result of one or more changes: (1) acceleration of transport, (2) increased glucose production, and/or (3) reduced phosphorylation. As regards

Johnson, 1955). The effect of insulin was relatively small in the *gastrocnemius* but was statistically significant ($P < 1 \cdot 0$). It seems probable that extracellular factors limit the supply of glucose to the cells of this muscle and thus reduce the magnitude of the insulin effect.*

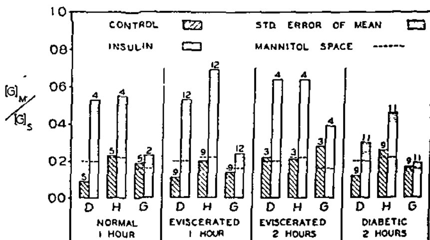


FIG. 8 The effect of insulin on the distribution of free glucose in the muscle of normal, eviscerated and alloxan diabetic rats.

Abbreviations: $[G]_M/[G]_S$, the ratio of the concentration of free glucose in the muscle water to the concentration in the blood serum water (see text for

0·1, heart < 0·1, gastrocnemius > 10.

Since evisceration can cause extensive circulatory and metabolic changes, it was important to test the effect of

utilization rises. With full insulinization and abundant substrate, the glucokinase reaction may become the rate-limiting step.

In alloxan diabetes, it was concluded earlier (Cori, 1946; Colowick *et al.*, 1947; Krahl and Cori, 1947) that inhibition of the hexokinase system is the cause of the reduced glucose uptake by muscle characteristic of this condition. The present observations suggest, however, that the immediate cause is an inhibition of glucose transport. With glucokinase inhibition, glucose entering the cell would accumulate as the free sugar in the absence of insulin. The present data, however, show that little or no intracellular sugar is present. This absence of intracellular glucose, as discussed earlier, is an indication that transport is the rate-limiting step for glucose uptake. The reduced uptake in diabetes must be due, therefore, to inhibition of this step. With insulin administration, transport is accelerated, as shown by the rise in intracellular free sugar. It has been shown earlier that diabetic muscle is somewhat resistant to insulin with respect to glucose uptake (Krahl and Cori, 1947; Villee and Hastings, 1949; Park, 1952). This could be ascribed to the smaller effect of the hormone on the transport process. It could possibly be due to a latent inhibition of glucose phosphorylation which could then restrict glucose utilization when the free sugar became available within the cell.

Effect of insulin on monosaccharide transport in the brain

In the case of the brain, insulin did not affect the distribution of any of the sugars tested, including glucose (Fig. 4). The mannitol space of the brain was very small, indicating that this alcohol probably does not pass the blood-brain barrier. Fructose and ribose had the same distribution and thus apparently also pass this barrier slowly, if at all. This would explain the observation that fructose is utilized poorly by the intact brain although it is an excellent substrate for brain hexokinase (Slein *et al.*, 1950) All the other sugars

insulin action, there are good reasons for excluding the last two. Firstly, glucose production by muscle is slight, due to the absence of glucose phosphatase and because the glucokinase equilibrium strongly favours glucose-6-phosphate formation. Secondly, in experiments with the isolated rat diaphragm (Park, 1953; Park *et al.*, 1955), we could find no effect of insulin on glucose production. The muscle was incubated in a medium containing ^{14}C-labelled glucose and, after an appropriate time, the free sugar in the tissue was estimated and counted. Insulin caused an increase in the quantity of glucose but did not reduce its specific activity. Thus the extra free glucose must have originated in the medium and not in unlabelled intracellular precursors. Thirdly, the possibility that phosphorylation would be reduced contradicts the well established fact that insulin increases glucose utilization, which necessarily goes through the glucokinase step. On these grounds, therefore, it seems reasonable to conclude that insulin accelerates transport of glucose through the membrane. This is consistent with the earlier conclusions in regard to galactose, mannose, fructose, L-arabinose, xylose and ribose.

The present studies indicate that this action of insulin is distinct from any effect on the glucokinase system, as postulated earlier (Cori, 1946; Colowick *et al.*, 1947). Firstly, insulin accelerates the transport of such sugars as galactose, L-arabinose and xylose which are phosphorylated very slowly, if at all, by this enzyme. Secondly, in the case of glucose, the intracellular free sugar would fall rather than rise if the primary action of the hormone were on the glucokinase system. It also follows, on the same grounds, that glucokinase is not an integral part of the transport mechanism. It would further appear that membrane transport is the rate-limiting step for glucose uptake and utilization by the non-insulinized muscle. This follows from the observation that no intracellular glucose is found in the absence of the hormone. The intracellular enzyme systems are therefore limited by the rate at which the transport step provides substrate. With acceleration of transport, more glucose becomes available and its

A model of membrane transport

LeFevre (1948, 1953, 1954), LeFevre and LeFevre (1952), and Widdas (1952, 1954) have shown that the entrance of glucose into the human erythrocyte is not a process of simple diffusion. They have proposed that glucose is transported through the cell membrane combined with a "carrier" molecule. This proposal, in somewhat modified form, is illustrated in Fig. 5.

Glucose, represented by G, is postulated to form a complex with some "carrier" molecule, X, at the outer surface of the cell. The complex, GX, passes through the membrane, a distance of 100–200 Ångströms (see Ponder, 1948), by thermal agitation or diffusion and releases free glucose at the inner surface. The process is reversible and may or may not involve enzyme catalysis. The rate of transport will always be limited by certain fixed properties of the system, such as the total number of carriers and their speed of movement through the membrane. Within these limits, however, transport will vary with the concentration of glucose, since this will determine the extent of complex formation.

From these postulates, the equations shown under Fig. 5 have been developed to predict the kinetics of transport. When the glucose concentration is appreciable on one side of the membrane and negligible on the other, transport is unidirectional and is described by Equations 6 and 7, which have the form of the Michaelis-Menten expression for enzyme-catalysed reactions

These equations predict that transport, T, will increase in non-linear fashion as the glucose concentration is raised. When the glucose concentration becomes high relative to the dissociation constant of the carrier complex, K_S, transport will approach an upper limit, T_{max}. By comparison, an equation based on simple diffusion would predict a linear relationship between concentration and rate. When glucose is present on [illegible]

passed the blood-brain barrier, and in some cases reached high enough distributions to indicate penetration of tissue cells. *In no case, however, did insulin accelerate their transfer.* This probably accounts for the fact that the hormone does not increase glucose utilization by brain tissue (see Himwich, 1951).

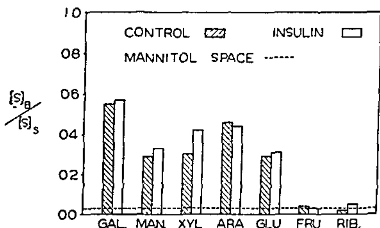


FIG. 4 The effect of insulin on the distribution of several hexoses and pentoses in the brain of eviscerated rats.

RIB, D-ribose.

Procedures: These data were obtained from the experiments outlined under Figs 1, 2, and 3.

In no case was the effect of insulin statistically significant.

Membrane Transport of Glucose in the Rabbit Erythrocyte

The localization of an effect of insulin to the transport of glucose across the membrane of the muscle cell has led us to investigate more closely the nature of the transport process. For technical reasons, which will become apparent later, we have used the rabbit erythrocyte for these studies although transport in this cell is probably not sensitive to hormones.

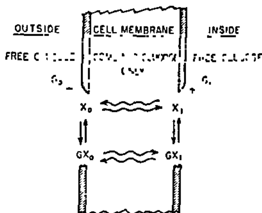
Inward and outward transport as a function of glucose concentration

We have measured transport as a function of glucose concentration and have compared these kinetics with those based on the above model. Our observations confirm and extend the earlier results of LeFevre and Widdas.

In the rabbit erythrocyte is was possible to measure inward and outward transport separately. To measure inward transport, advantage was taken of the fact that the hexokinase activity is high in this cell relative to the transport rate and ordinarily maintains a low level of intracellular glucose. In the presence of methylene blue, hexokinase activity is increased still further, and the intracellular glucose is reduced to a negligible level even with near-maximal transport. Under these conditions transport is the rate-limiting step for glucose utilization. Inward transport could be measured, therefore, by determining glucose disappearance when the cells were incubated at various external glucose concentrations with methylene blue. The curve obtained (Fig. 6) has the form expected with kinetics of the Michaelis-Menten type.

To measure outward transport, the cells were prepared as follows. A preliminary incubation was carried out in a high concentration of glucose and 5×10^{-3} M-fluoride. The erythrocytes now accumulated intracellular glucose, since the fluoride completely stopped glucose utilization but did not interfere with transport. The cells were then washed at 0° until free of external glucose with no appreciable loss of intracellular sugar because of the high temperature coefficient for transport, about 2·5 per 10°. They were then placed in a large volume of glucose-free medium containing yeast hexokinase and ATP to trap glucose appearing on the outside. When the suspension was warmed to 37°, transport outward became active and was measured as a function of the internal glucose concentration. As shown in Fig. 6, the relationship of outward transport to concentration was essentially the same as in the case of inward transport.

The constants for these curves, which correspond to the



G = GLUCOSE
X = UNOCCUPIED "CARRIER"
GX = OCCUPIED "CARRIER"
o = ON OUTSIDE SURFACE
i = ON INSIDE SURFACE
⇄ = REACTION CLOSE TO EQUILIBRIUM
= RATE-LIMITING PROCESS

Fig. 5. A model of glucose transport across the erythrocyte membrane (see text for discussion).

The derivation of the transport equations based on this model is as follows:

$\frac{[G]\,[X]}{[GX]} = K_S$. K_S is the same at both surfaces so that transport has the same kinetics whether inward or outward

4. To find [GX], use $[GX] + [X] = [C]$ and $K_S = \frac{[G]\,[X]}{[GX]}$ as in the Michaelis-Menten equation The result is: $[GX] = \frac{[C]\,[G]}{K_S + [G]}$.

5

6

out and $\quad {}_oT_i = T_{max}\left(\frac{[G_o]}{K_S + [G_o]}\right)$.

7. Similarly, for outward transport, ${}_iT_o$, when $[G_o] = 0$, the first term in brackets drops out: $\quad {}_iT_o = T_{max}\left(\frac{[G_i]}{K_S + [G_i]}\right)$.

K_S of the postulated glucose-carrier complex and the T_{max}, were determined from the Lineweaver-Burk (see Lardy, 1949) plot shown in Fig. 7. It should be noted that a diffusion process would also give a straight line when plotted in this manner, but the line would pass through the origin.

The model was then tested in the more complex situation in which glucose is transported in both directions simultaneously. This is the case when cells incubated in glucose

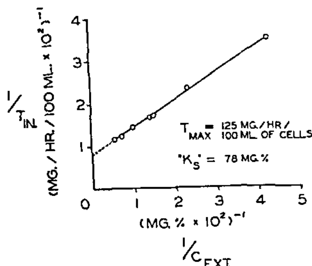


FIG. 7. Inward glucose transport in the erythrocyte plotted according to Lineweaver and Burk (see Lardy, 1949) The interpretation is given in the text.

and fluoride accumulate intracellular sugar. Using the constants determined above, the time curve of accumulation could be predicted by integration of Equation 5. When the course of glucose accumulation was determined directly, the predicted and experimental curves were in good agreement, as seen in Fig. 8. These curves are helpful in explaining the well known delayed osmotic haemolysis of erythrocytes in high concentrations of glucose. With a high external glucose

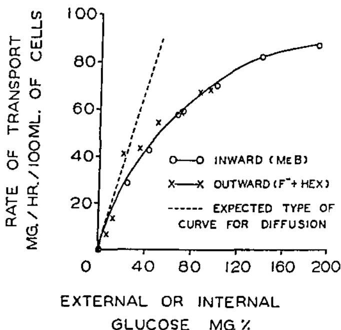


FIG. 6 Inward and outward glucose transport in the rabbit erythrocyte as a function of the glucose concentration (see text for interpretation).

Inward transport is shown as a function of the *external* glucose concentration when the internal concentration is negligible (< 2 mg per cent)

Outward transport is shown as a function of the *internal* [illegible]

this type, we determined the transport of a number of sugars and sorbitol into fluoride-treated cells. As shown in Fig. 9, all sugars tested entered more rapidly than glucose. It should be emphasized, however, that a comparison of transport rates

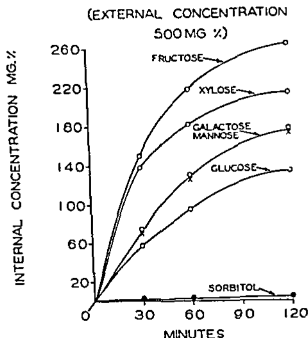


FIG. 9. The accumulation of several monosaccharides and sorbitol in fluoride-treated rabbit erythrocytes

A 10 per cent (v/v) suspension of cells was incubated at 37° and pH 7 4 with the substance indicated at a concentration of 500 mg per cent

might show quite different results at other external sugar concentrations * The alcohol sorbitol, which is similar to these sugars in size and solubility, did not enter the cell at all. This

* According to the present kinetic analysis, it would be predicted that the sugar whose carrier dissociation constant was nearest the value of the external sugar concentration would approach equilibrium across the membrane most rapidly.

concentration, inward transport is near a maximum rate. After only a moderate rise in internal concentration, outward transport will approach closely the same maximum. The net transfer of glucose across the membrane will then be small although a large concentration gradient still remains.

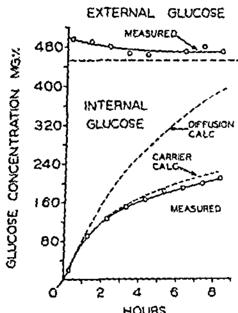


FIG. 8. The accumulation of glucose in fluoride-treated rabbit erythrocytes (see text for interpretation).

Competition among sugars for the transport system

LeFevre (1954) and Widdas (1954), by showing that certain sugars compete for entry into the human erythrocyte, obtained substantial support for the postulate that chemical combination is requisite to transport. As a preliminary to studies of

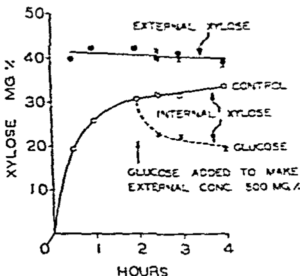


Fig. 11. The extrusion of xylose from the erythrocyte against a concentration gradient due to competition by [illegible]

of the cell, although this movement took place against a concentration gradient. Similar results were obtained when glucose was added to cells equilibrated with galactose. This "pumping out" of xylose can be explained as follows. At the external surface of the cell, glucose competes successfully for transport and reduces the inward movement of xylose. Inside the cell, however, glucose is metabolized and does not compete. Outward xylose transport therefore continues at

is further evidence that the membrane does not have physical openings through which these substances can pass.

As shown in Fig. 10, glucose and galactose were competitive with respect to membrane transport. Glucose was the more effective competitor, indicating that it had a higher affinity

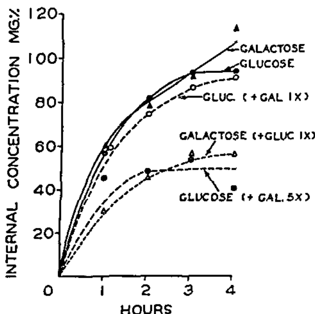


FIG 10 The competition between glucose and galactose for transport in rabbit erythrocytes treated with fluoride.

The intracellular accumulation of the sugar is a measure of the transport rate.

The external concentrations of the sugars were 200 mg per cent or multiples of this figure as indicated.

for some binding site in a common transport system. Similar data were obtained for glucose v. xylose, but not for glucose v. fructose.

Widdas (1952) predicted that competition for a "carrier" would result under certain conditions in the transport of a sugar against a concentration gradient. An experimental verification of this proposal is presented in Fig. 11. Fresh cells

were first incubated in a low concentration of xylose which is metabolized slowly, if at all. At the end of two hours the internal xylose concentration closely approached the external level. When, at this point, glucose was added to the external medium, the internal xylose fell rapidly to a lower level. Since the xylose was not metabolized, it must have moved out

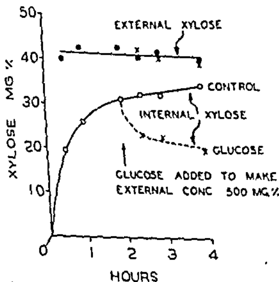


Fig 11

of the cell, although this movement took place against a concentration gradient. Similar results were obtained when glucose was added to cells equilibrated with galactose. This "pumping out" of xylose can be explained as follows. At the external surface of the cell, glucose competes successfully for transport and reduces the inward movement of xylose. Inside the cell, however, glucose is metabolized and does not compete. Outward xylose transport therefore continues at

the original rate and exceeds inward transport until a new equilibrium is reached. The energy for moving xylose against a gradient is supplied to the transport system by the transport of glucose *from a region of higher to lower concentration.* The metabolism of glucose serves to maintain this concentration gradient but does not supply energy directly.

Concluding Remarks

From the present studies certain differences are apparent in the transport of monosaccharides among cells from various tissues. In muscle, all sugars examined entered the cell at appreciable rates, and their transport was accelerated by insulin in all cases. In the brain, presumably at the blood-brain barrier, fructose and ribose were transported very slowly, if at all, whereas several other sugars passed this barrier readily. Transport into the brain was not effected by the hormone. In the erythrocyte, all sugars studied were transported into the cell, but at apparently different rates than in the muscle or brain. No effect of insulin was observed in this cell.

It is apparent, from our own and other studies, that glucose transport may vary widely in relationship to the rate at which the sugar is utilized by intracellular enzymes In the human erythrocyte, transport is so fast that the intracellular and extracellular glucose concentrations are virtually equal at all times. Here the activity of the intracellular enzymes is rate-limiting for glucose utilization. On the other hand, in the non-insulinized muscle, transport is slow relative to utilization, and the concentration of intracellular glucose is negligible. In this case transport is the rate-limiting step for glucose utilization and as such is an appropriate site for the acceleratory action of insulin. Furthermore, the transport process is presumably accessible to insulin without penetration of the cell.

There are indications, on the other hand, that the transport process has certain similar properties in all the cells examined

in this study. It is clear that transport is not a process of simple diffusion but involves a physicochemical combination of the sugar with some component of the cell membrane. The product of transport, however, is the free sugar, and phosphorylation in the usual sense is not involved. The energy for transport is derived from the concentration difference across the membrane and not from intracellular metabolism directly. Intracellular metabolism, however, may serve to maintain a concentration gradient. In this way, furthermore, it can make transport virtually a unidirectional process by rapid removal of the transport product.

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DISCUSSION

Schulze: The mechanism of acceleration of the transport and the penetration of glucose through the cell membrane is obviously an essential part of the principle increasing the peripheral sugar utilization and the work economy of muscles in diabetics when treated with insulin. I [illegible]

[illegible] treated with insulin their restitutional quotient gets closer to the age norm of healthy individuals, as shown by Fig. 2.

Ross: I am really an interloper in this field as I approached it from an entirely different aspect. At the Institute of Ophthalmology we were interested in the rates at which various substances, especially anti- [illegible]

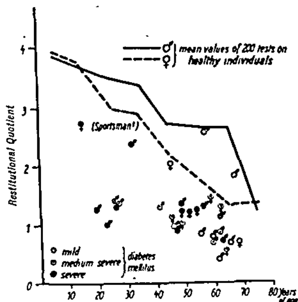


FIG 1 (Schulze). Restitutional quotient = $\frac{O_2 + \text{consumption during effort}}{O_2 + \text{consumption during recovery}}$, a measure of physical efficiency in healthy and diabetic persons, in relation to age.

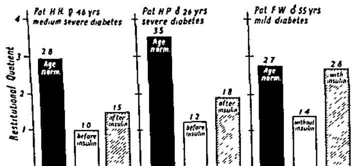


FIG. 2 (Schulze). Amelioration of physical efficiency in diabetics by insulin therapy, as proved by increase in restitutional quotient

the exception of glucose (Fig. 1). Now *glucose is very water-soluble* but very ether-insoluble and in theory should have a very slow rate of

barrier between the blood and the intraocular fluids is a very specific one. *Urea, for example,* a very small molecule, has a slow rate of entry; *in other words, entry is obviously not through pores.* The barrier is not

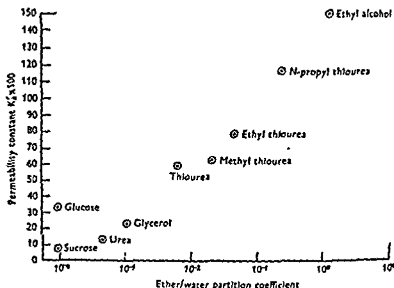


FIG. 1 (Ross) The rate of penetration of non-electrolytes from the blood into the aqueous humour in rabbits
(Ross, E. J. (1951). *J. Physiol.*, **112**, 229)

just a capillary wall but is a very selective membrane. So here is a system where the permeability of a cell membrane can be readily

words, insulin had very considerably accelerated the entry of glucose into the eye. To obtain further proof of this, I took alloxan diabetic rabbits, and in these the rate of entry ranged from 0·74 to 1·37, i.e., there was a reduced rate of entry in these animals. An interesting point is that in the alloxan diabetic animal during the hypoglycaemic phase that follows alloxan administration the transfer constant was

large doses gave a distinct reduction of the rate. These were intact animals.

Young: And this was so even though the insulin was believed to be free from glucagon. I think that is important.

Ross: I used Novo insulin, completely free from glucagon.

C. F. Cori: Are these amounts per kg. or per ml?

Ross: No.

Lawrence: That remains to be done.

Young: Prof. Candela, have you not some evidence about high blood insulin values in the hypoglycaemic phase after the administration of alloxan?

R.-Candela: Yes, I have some results. During the hypoglycaemic phase after administration of alloxan we found more plasma-insulin

I have done some work with lenses and found that insulin increased the rate of uptake of glucose by about 350 per cent. With galactose there [illegible]

glucose, shows a relatively high temperature coefficient, appears to be enzymic in character in that it follows the Michaelis-Menten law, and has a large Michaelis constant for the two hexoses. In addition, there was a small influence of insulin on the penetration of free sugar into the tissue, but this could be demonstrated equally at 0°, 10° and 37°, and appeared to be an increase in the diffusion of the external sugar into the available space of the muscle. This effect seemed of little importance in determining the increased uptake of sugar caused by insulin. We were tempted to identify the insulin-sensitive process with the hexokinase reaction, mainly because of the results obtained with fructose. As first shown by Mackler and Guest (1953, *Proc Soc. exp. Biol*, *N.Y.*, 83, 327), the uptake of this sugar by the diaphragm is partly inhibited and its stimulation by insulin completely inhibited, by sufficient amounts of glucose, suggesting the existence of two pathways: one via hexokinase and insulin-sensitive, the other via fructokinase and unaffected by insulin. An active transfer process of the type postulated by Prof. Park could of course equally be involved, provided it possessed similar kinetic pro- [illegible]

hexokinase is bound in the intact tissue in a manner which lowers its affinity for its substrates. Obviously it will require more experiments to settle this matter.

Park: The idea that insulin may accelerate a process of physical diffusion, as suggested by Profs. Cori and de Duve, does not seem to me [illegible]

C. F. Cori: We have found that pentoses go into muscle at a very slow rate. I do not know whether they are transported by an active process, but in general if there is an active process that is one that makes the substance go in rapidly. I do know that insulin and muscular work have the same effect on the distribution of pentoses. This distribution is not competitively inhibited by other sugars, and it does not have the character of an active transport, or else there are two distinct transport systems, one for glucose and one for the pentoses. The cell membrane is not static but presumably adapts all the time to the metabolism that is going on inside the cell. Muscular work, through a change in the metabolism inside, may influence the permeability of the membrane. Insulin may act in a similar manner with respect to the pentoses, that is, a changed metabolism inside may change the property of the membrane.

PANCREATIC ISLETS AND GROWTH

CESARE CAVALLERO
Institute of Pathological Anatomy, University of Milan

IN RECENT years, good evidence has been obtained that pancreatic islets are concerned not only with the production and secretion of insulin, but also of another hormonally active substance, called glucagon in the early literature and described as hyperglycaemic-glycogenolytic factor (HGF) in most of the recent papers.

Today, the existence of two pancreatic internal secretions seems to be well established; it is generally accepted that insulin is secreted by the β-cells of the islets, whereas there is good reason to believe that glucagon is a product of the α-cells. Since glucagon can be extracted from fish insular corpuscles showing only endocrine structures (Malandra and Mosca, 1952), and from the mammalian duct-ligated pancreas with atrophic acinar tissue (Sutherland and de Duve, 1948; Cavallero and Malandra, 1951), as well as from the alloxanized and duct-ligated pancreas (Gaede, Ferner and Kastrup, 1950), its origin in exocrine tissue or in insular β-cells seems to be hardly maintainable.

The research work of the Lilly group (Staub, Sinn and Behrens, 1953) has firmly established the chemical individuality of this new principle, besides making it available in pure crystalline form for biological study.

Two main properties have been attributed to glucagon, namely, a regulatory effect on the carbohydrate metabolism, and a general effect on the processes of growth. As regards the first point, it was claimed that this second pancreatic hormone might play an important rôle in the disturbances of carbohydrate metabolism and particularly in the pathogenesis of human diabetes mellitus. Undoubtedly, glucagon is a very

active glycogenolytic and hyperglycaemic substance, and, in this way, it might counteract some effects of insulin; however, this fact does not necessarily imply that it should be considered as a diabetogenic factor.

Our experimental work on this particular aspect has given rather disappointing results, since neither in rats fed *ad lib.*, nor in rats force-fed a carbohydrate-rich diet, has glucagon shown any evident diabetogenic activity. We could only demonstrate that this pancreatic principle is able to potentiate the effects elicited by the so-called diabetogenic factors, such as cortisone, corticotrophic and somatotrophic hormones; combined treatment of glucagon and either of the above-mentioned hormones, if given at subdiabetogenic dose levels, resulted in a temporary diabetes with insulin resistance (Cavallero and Malandra, 1953; Cavallero, Malandra and Galansino, 1954).

Similarly, Ingle, Beary and Purmalis (1954) could not obtain glycosuria in normal rats even by continuous administration of glucagon, and Galansino and co-workers (1955) were unable to cause glycosuria or alter the glucose tolerance of chronically treated rats.

A relationship between pituitary somatotrophic hormone and glucagon has also been indicated and it has been suggested that the growth-promoting effect of the pituitary hormone could be mediated through glucagon (Bornstein, Reid and Young, 1951; Cavallero and Mosca, 1953; Foà *et al.*, 1953). It has been claimed that this pancreatic factor can increase the width of the epiphyseal line in hypophysectomized rats (Elrick, 1953), but this statement was not confirmed by other investigators (Geschwind and Staub, 1953; Mitchell, Rice and Girerd, 1954). On the other hand, skeletal growth and weight increase have been reported by Salter and Best (1953) in hypophysectomized rats fed *ad lib.* and receiving gradually increasing doses of protamine zinc insulin, and similar results have been obtained by Smith, quoted by Young (1953), giving insulin plus glucagon.

Since most preparations of insulin are known to be

contaminated by glucagon, it has been objected that the effects on growth of the hypoglycaemic hormone might be ascribed to this contamination; reciprocally, an alleged activity of glucagon on growth has been attributed to insulin contamination.

The morphological approach to this important problem has given contrasting results, since neither hypophysectomy nor administration of somatotrophic hormone appears to be able to produce any definite and constant change of the islets. If given to hypophysectomized rats or to pituitary dwarf mice with free access to food, the pituitary hormone increases the area and the volume of the islet tissue, but this does not seem to be the case if it is given to animals on restricted food intake (Cavallero and Dova, 1954; Mosca, 1954). The somatotrophic hormone appears also to be unable to affect the relative percentage of α- and β-cells, both in young and adult hypophysectomized rats (Mosca, 1954).

The best morphological evidence that glucagon might be concerned with the processes of growth is given by the fact that the α-cells, which are allegedly the site of production of this hormone, are present in high numbers in the islets, both of man and other mammals, during foetal and early life, when the growth is very active, whereas their number decreases consistently as soon as the body growth stops or slows down.

Effect of Glucagon on Body Growth of the Chick Embryo

Our first experimental approach to the possible effect of glucagon on growth was carried out on Leghorn chick embryos, using a preparation of amorphous glucagon (Lilly Laboratories, lot 208–108 B–234) which, according to the manufacturer, contained 0·005 unit of insulin per mg. Two experimental groups of eggs were injected in the chorio-allantoic membrane at the ninth day of incubation, with 5 and 25 μg. of glucagon respectively, dissolved in Tyrode

solution at pH 9·5; two control groups were respectively injected with Tyrode alone and with $1{\cdot}25 \times 10^{-4}$ units of regular Novo insulin, free from hyperglycaemic activity, i.e. with the dose of insulin contaminating 25 µg. of glucagon. The eggs were sacrificed in groups at the fourth, seventh and ninth day after the injection and the fresh embryos weighed.

Table I

MEAN BODY WEIGHT IN G. OF FRESH CHICK EMBRYO TREATED WITH DIFFERENT HORMONES AT THE 9TH DAY OF INCUBATION

Number of embryos	*Day of incubation*	*Controls*	*Insulin-treated*	*Glucagon-treated*	
				5 µg.	25 µg.
40	13th	6 3335	5 7223	7 0673	7 1185
40	16th	11 7213	10 9018	14 2544	14 4624
40	18th	16 3643	15 9238	19 5051	18 9632

From the results, summarized in Table I, it appears that both doses of glucagon have been effective in stimulating the growth of the chick embryo. On the contrary, pure insulin has failed to obtain any such effect. Biochemical determinations of the humidity, total fat and nitrogen content of the whole embryos have given inconstant results; in general, there was some tendency towards an increase of protein and water content of glucagon-treated embryos, but the statistical analysis of the data showed no significant differences.

Effect of Glucagon, Insulin and Somatotrophic Hormone on the Growth of Pituitary Dwarf Mice

It has been claimed that pituitary dwarf mice constitute a useful animal material for experimental investigations concerning the function of the pituitary as a superior centre of growth processes. The strain of black-silver dwarf mice studied by Snell (1929) is mainly characterized by a hereditary

hypoplasia of the anterior pituitary; it should be underlined that in these animals there is a pure growth-promoting insufficiency, whereas, unlike the hypophysectomized animals, the pituitary corticotrophic function appears to be unaffected. Investigations into the reactions of dwarfs to the treatment with hormones from the pituitary and other endocrine glands have shown the extreme sensitivity of this animal to several growth-stimulating preparations, such as pituitary somatotrophic and thyrotrophic hormones, prolactin and thyroxine (Kemp and Marx, 1937; Fønss-Bech, 1947).

In previous comparative studies between dwarf and normal mice of the same strain, we have observed that the anterior pituitary hypoplasia of dwarfs is associated with some islet abnormalities, such as a lower absolute amount of endocrine tissue, a numerical predominance of small islands, and a higher percentage of α-cells. Moreover, hypotrophic changes, both of α- and β-cells, are evident and have been considered as a morphological sign of a deficient insulin and glucagon secretion.

Prolonged administration of pituitary somatotrophic hormone (STH) is very active in producing an increase of weight and length in pituitary dwarfs; concurrently, there is an absolute increment of the islets, associated with hypertrophic changes of the cells and a tendency for the α : β-cell ratio to reach normal values (Cavallero and Dova, 1954).

The main morphological differences between dwarf and normal mice concern the weight and size of the body, as well as the tail length; in dwarfs, all organs are distinctively underdeveloped. Besides, we have been able to ascertain by biochemical studies other important differences in body composition, which appear to duplicate, at least in part, those seen in hypophysectomized animals.

In fact, the empty carcass of the pituitary dwarf mouse [illegible]

whereas fat is present in low amount (Table II). This bio-

Table II

AVERAGE COMPOSITION, IN PER CENT OF FRESH TISSUE, OF EMPTY CARCASSES AND LIVERS OF DWARF AND NORMAL MICE FED *ad libitum*

Number of determinations in parentheses

	Carcass		*Liver*	
	Dwarf (10)	*Normal* (10)	*Dwarf* (10)	*Normal* (10)
Water	60 32	61·12	68 27	67·25
Fat	17·98	14 21	8 04	7 02
Protein	19 75	22 71	16·12	19 76

chemical pattern is quite similar to the one we observe in hypophysectomized animals. However, dwarfs, when fasting, do not behave like hypophysectomized animals, as they show a tendency to maintain high glycogen levels, both in liver and muscle, and to increase to a lesser degree the liver fat (Table III). This discrepancy might be accounted for by the normal adrenal function or by the above-mentioned insular deficiency in pituitary dwarfs.

Table III

INFLUENCE OF FASTING ON LIVER AND MUSCLE GLYCOGEN AND ON LIVER FAT OF DWARF AND NORMAL MICE; AVERAGE VALUES IN G. PER CENT OF FRESH TISSUE

Number of determinations in parentheses

	Dwarf		*Normal*	
	Fed (10)	*Fasted* (9)	*Fed* (10)	*Fasted* (9)
Liver glycogen	3 951	0 560	2 366	0 440
Liver fat	3 040	10 300	7 020	19 380
Muscular glycogen	0·826	0 410	0 648	0·229

In summary, the general metabolism in these animals appears to be clearly disturbed, mainly with regard to the mobilization of fat from peripheral depôts and the utilization of the stored glycogen.

In a first experimental series, the effect of a preparation of amorphous glucagon (Lilly Laboratories, lot 208–158B–197) on the body weight of pituitary dwarfs was investigated. The preparation employed was contaminated by insulin, 0·005–0·05 unit per mg., and its purity, based on the potency of

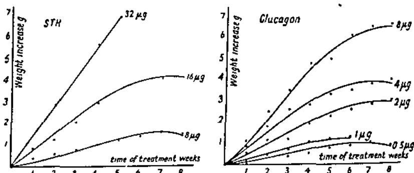


FIG 1. Mean weight increase of dwarf mice treated with various doses of STH and glucagon.

crystalline glucagon, was approximately 50 per cent. The substance, prepared in buffer solution at pH 9·5, was injected daily subcutaneously in dwarf mice, 6–8 weeks old and weighing 4–6 g. The animals had free access to a carbohydrate-rich diet.

During treatment a consistent and gradual increase in weight was observed, a well defined effect being already evident with a dosage of 0·5 µg. per day (Fig. 1). Over the range studied, body-weight increase bore an approximately linear relationship to the amount of glucagon given (Fig. 2).

Similar experiments have been undertaken with purified STH (Armour, lot R285–183) and protamine zinc insulin (PZI, Lilly). Dose levels of STH similar to those of the glucagon group were assayed; the daily dose of PZI was, at the

start, 0·05 unit and was then increased to 0·1 unit. Since insulin appeared to be fairly well tolerated by the hypopituitary animals, the effect of regular Novo insulin, free from hyperglycaemic activity, was also investigated at a dose of 0·005 unit, increased later to 0·01 unit once daily.

Obviously, STH treatment caused body-weight increase, the minimal effective dose being 2 µg. daily; the relative potency of STH was found to be approximately a third of that of the glucagon preparation employed (Fig. 1).

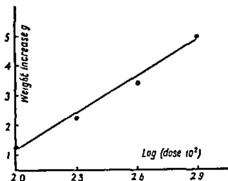


FIG. 2 Dose-effect response of pituitary dwarf mice treated for 5 weeks with different doses of glucagon.

Both regular insulin and PZI raised the weight of dwarfs, but this effect was clear-cut only at the beginning of treatment and later with increasing doses; thus, this effect did not appear to be proportional to both dose and time of treatment (Fig. 3).

Comparison of the effect of the three preparations tested shows that the gain in weight and length was evidently higher in STH- and glucagon-treated dwarfs than in the insulin group. Besides, in the former groups the tail growth, compared to the body growth, was relatively higher than in the latter.

At necropsy, it was observed that STH and glucagon treatment results in a tendency of all organs studied, except the adrenals, to reach the normal weight values. Similar changes,

but to a lesser degree, have been observed in the liver and testicles of the insulin group, whereas the spleen, thymus, pancreas and kidney weights were within the normal range and the adrenals were consistently increased (Table IV).

At the histological examination, the main changes observed concern the endocrine pancreas, the interscapular adipose tissue and particularly the bones. Full maturity of the islet

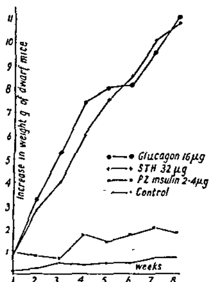


FIG. 3 Representative growth curves of untreated and of hormone-treated dwarf mice.

apparatus was remarked in STH and glucagon groups; on the contrary, insulin-treated animals showed hypotrophic islets and a high number of α-cells, like untreated dwarf mice. The interscapular adipose tissue was found to be modified only in the insulin group, where the granular and finely vacuolated pattern of the cell was lost and replaced by large vacuolar cytoplasmic structures (Fig. 4).

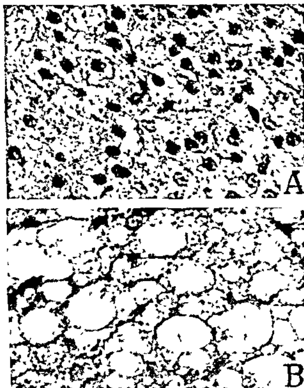


FIG 4 Interscapular adipose tissue of untreated (A) and insulin treated (B) dwarf mouse

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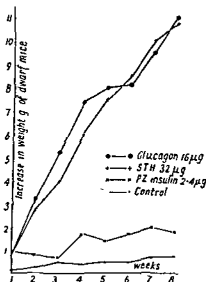


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and diaphysis associated with a good differentiation of the epiphyseal cartilage was evident. The insulin-treated dwarfs behaved quite differently, showing only minor or no changes (Fig. 5).

Finally, important biochemical changes in carcass and liver composition of the three experimental groups have been ascertained. In this investigation the total fat has been determined on aliquots of dry tissues by the gravimetric method

Table IV

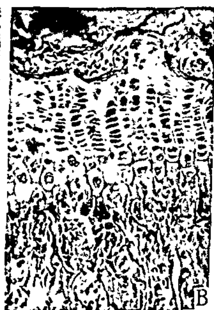
ORGAN WEIGHTS, IN PER CENT OF BODY WEIGHT, OF NORMAL, UNTREATED AND TREATED DWARF MICE OF THE SAME AGE; TREATMENT WITH HORMONES LASTED 8 WEEKS; 10 ANIMALS PER GROUP

Organ	*Normals*	*Untreated dwarfs*	*Treated dwarfs*		
			STH 16 μg /day	*Glucagon* 8 μg./day	*PZ insulin* 0 05–0·1 *I.U /day*
Liver mg.	5063	3116	4900	4865	4495
Kidney mg.	1453	1154	1181	1235	1005
Spleen mg	356	161	450	408	190
Thymus mg.	187	153	244	182	156
Heart mg.	559	616	523	577	403
Pancreas mg.	507	203	274	313	213
Testicles mg.	652	209	366	296	308
Adrenals mg	20 30	21·10	21 10	22·70	25 10
Body weight g	17·52	4·47	10 15	11 98	7 32
Body length cm	16 48	9 07	12 10	11 77	10·37
Tail length cm.	7 75	3 86	5 58	5 33	4 61

and the total nitrogen content on defatted and digested tissues by the micro-Kjeldahl technique; the glycogen content has been evaluated on fresh tissues by the Good, Kramer and Somogyi method.

In fed animals, the main biochemical changes elicited on body composition by glucagon appear to duplicate exactly those determined by STH. Actually, the mean composition of the empty carcass showed a higher water and protein content and a significant loss of fat; concomitantly, the liver









[illegible] pituitary dwarf mice under hormonal treatment: [illegible]

animals of the [illegible]

fat was increased in both groups. Besides, chronic treatment with glucagon resulted in an increase of liver glycogen, which was lacking in the STH group. The gain in weight of the animals treated with glucagon or STH appears, therefore, to be due prevalently to an increase of protein and water.

The biochemical changes determined by insulin have been quite different; the water content was consistently reduced and, correspondingly, we have observed an increase of fat and protein, both in carcass and liver. The glycogen content of the liver was lowered (Table V).

In fasted animals, the effects of glucagon and STH have not been identical, since STH increased the liver fat, whereas glucagon diminished it. As far as liver and muscle glycogen are concerned, these two hormones behaved accordingly and failed to give patterns dissimilar from those of the control fasted dwarfs (Table VI).

Table VI

EFFECT OF FASTING ON LIVER AND MUSCLE GLYCOGEN AND ON LIVER FAT OF UNTREATED AND TREATED DWARF MICE, AVERAGE VALUES IN G PER CENT OF FRESH TISSUE

Number of determinations in parentheses

Group		*Liver glycogen*	*Muscular glycogen*	*Liver fat*
Controls	fed (10)	3 051	0 826	3 04
	fasted (9)	0 560	0 410	10 30
Glucagon	fed (13)	5 530	0 703	6 08
	fasted (8)	0 516	0 358	4 44
STH	fed (10)	3 611	0 936	6 08
	fasted (8)	0 491	0 335	12 11

Comment

Under the conditions of our investigations, glucagon has been shown to be an active growth-stimulating factor. In embryonic life, as well as in pituitary dwarfism caused by a pure deficiency of pituitary growth hormone, it can induce a

Table V

AVERAGE CHANGES IN BODY COMPOSITION OF FED DWARF MICE TREATED WITH GLUCAGON, STH OR INSULIN FOR 4–8 WEEKS

Number of animals	*Group*	*Terminal body weight g.*	*Carcass composition %*			*Liver composition %*			
			Water	*Fat*	*Protein*	*Water*	*Fat*	*Protein*	*Glycogen*
11	Control	5 2	60 32	17 98	19 65	68 27	3 04	16 12	3 051
13	Glucagon 8–2 µg.	12·1	64·22	13 09	21 03	69 94	6·08	15 81	5·530
10	STH 16–6 µg.	12 0	66 78	9·63	21 00	70 51	6·08	15·37	3·611
9	Insulin* 0·005–0 1 I.U.	6·8	52·20	24 03	22 93	67·70	8 98	17·12	2 927

* Three animals treated with regular insulin and six with PZI.

view that this hormone should be considered as a growth factor. However, owing to the fact that insulin causes modifications of the water and fat content of the body, unlike the effects elicited by somatotrophic hormone and glucagon, the problem arises whether growth might be accomplished through different mechanisms.

Acknowledgement

We are greatly indebted to Prof. T. Kemp, Director of the Institute for Human Genetics, University of Copenhagen, for supplying us with the strain of pituitary dwarf mice used in this investigation. Our co-workers, Drs. Malandra, Dova, Mosca, Fior and Galansino, have collaborated in carrying out the research. For material used in the experiments reported in this paper we are indebted to the following: Dr. W. R. Kirtley, of the Lilly Research Laboratories, for amorphous glucagon, and Dr. L. Steelman, of the Armour Laboratories, for growth hormone.

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well sustained growth. This is a "true growth", since not only are the body and organ weights concerned, but also an increase in body length and skeletal changes indicative of an active bone growth.

Furthermore, glucagon appears to have a high metabolic activity, since in pituitary dwarfs it has effected marked changes in body composition, similar to the ones elicited by pituitary somatotrophic hormone. This fact gives further support to the view of a close relationship between these two hormones.

Our research suggests that the main biochemical activity of glucagon concerns fat metabolism, namely, the mobilization of fat from the peripheral depôts and its oxidation in the liver; in agreement with other investigators (Payne, 1954; Haugaard and Haugaard, 1954), we have found that this principle appears to be an active fat-mobilizing factor and possibly *increases fat oxidation in the liver during fasting.* From these points of view, its action is opposite to that of insulin.

It is noteworthy that the growth-promoting effect of glucagon has been seen in animals in which the corticotrophic activity is unaffected. This fact might possibly explain how, in other tests, glucagon has been found to be inactive, and, on the other hand, the question arises of a possible supporting or "permissive" action of the cortical secretions on the glucagon activity.

As far as the effect of insulin on growth is concerned, both regular glucagon-free insulin and protamine zinc insulin, at [illegible] hormone, and not accompanied by any clear-cut bone proliferation.

The well demonstrated activity of insulin on nitrogen-retention of the *hypophysectomized rat (Lawrence, Salter and Best, 1954)*, as well as our observations that in dwarf mice it can increase the nitrogen content of the body, support the

growth, it was reported by Elrick (Elrick, H. ([illegible]). Proc. Soc. exp. [illegible]

to observe any effect whatsoever of highly purified glucagon on growth in [illegible]

about many interesting metabolic effects of glucagon, from Sutherland, Candela, de Duve and Cavallero The conditions under which these various studies have been conducted are, of course, quite different and the results of each one have eventually to be related to physiological conditions.

As far as growth is concerned, the report of Dr. Cavallero [illegible]

ing. I am not sure that Dr. Cavallero showed us the comparable width of the epiphyseal cartilages with the various hormones, with glucagon, somatotrophin and with insulin. One can see the change that he pointed out, but not a comparison of the exact width under comparable conditions. The mouse, as Dr. Cavallero said, is quite a different preparation from the hypophysectomized animal

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DISCUSSION

Best: There are, of course, many different aspects of growth and all of them are interesting. The question is often asked: is it true growth?

there are other very useful ones.

motes growth in hypophysectomized alloxan diabetic rats but in these Houssay animals somatotrophin has no effect on body weight. However, Houssay rats treated with both growth hormone and insulin gain

Following reports

Randle: Prof. Best, were the animals receiving thyroxine given extra thiamin?

Best: In Salter's work a synthetic diet was used. We thought that it was complete but it apparently lacked some component of the stock diet since with the latter the thyroxine effect was consistently obtained.

Sutherland: Dr. Cavallero, did the glycogen which was formed after

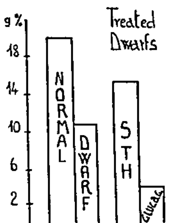


FIG 2 (Cavallero). Mean fat content of the liver, in g per cent of fresh tissue, in fasted and untreated normal and dwarf mice, and in fasted and STH- or glucagon-treated dwarf mice.

still remains above normal values. This fact may be observed both in

measurement. STH and glucagon treatment result in a similar effect, namely, determining a differentiation of a cartilaginous disc.

Now, may I ask, Professor Best, whether you have studied the effect

physectomized rats?

also.

Young: You have not given ACTH?

Best: No.

ause

Biol.

e an

extensive study of the question and we had some data too on the same subject. We have studied the sensitivity to steroids of the ossification

CHAIRMAN'S CLOSING REMARKS

sure that many of us have found that much interesting and perhaps valuable information has come from the work with which we were not particularly familiar.

present time.

Dr. Foà's introduction of prolactin into the discussion was an interesting one. Prolactin has lain fallow for a good many years, but I think many of us have felt all the time that it had some metabolic effect which was awaiting elucidation. I think Dr Foa will agree with me that much remains to be elucidated, but a start has been made on a matter of great interest, and I imagine that in the next year or two prolactin will become much more fashionable than it has been during the past few years.

I, in particular, was very glad to hear Dr. Goldner's views expressed so ably by him. He does not agree with some others, but I think the opportunity of hearing the discussion and learning more about his ideas has been extremely valuable to us all.

normal. The dwarf is obviously not

Ferner: From a morphological point of view we can propose a relation-

range for the adult.

activity of blood collected simultaneously from the umbilical cord of infants at birth and from the mother. No insulin activity was detected in the cord blood, but there was insulin activity in the maternal blood (McCance, R. A., Santos, R. F., and Randle, P. J. (1955), *Nature, Lond.*, 176, 115).

G. Cori: Prof. Ferner, how long does this preponderance of α-cells persist in extra-uterine life?

Ferner: This was a picture of an islet typical of the second half of foetal life. After birth there is a slow diminution of α-cells so that the normal picture is reached in adolescence.

Best: Prof. Ferner's slide would make a striking contrast with one showing the islets of an infant born of a diabetic mother, where you have a preponderance of β-cells.

Ferner: Yes, in the islets from the foetus of a diabetic mother there

* * * * * *

The possibility of explaining the action of hormones in terms of influences on enzyme systems is one that has seemed to recede into the background at times. Prof. Sutherland put this matter right in the foreground with respect to one system, but in many instances we are being driven back into experiments with intact cells, and even with the intact animal, in order to obtain a lead in the elucidation of the mechanism of hormone action.

long since passed, and thus very confusing overlap of actions of hormones seems unlikely to be merely the result of the contamination of one hormone by another. The possibility that biologically significant amounts of hormones lurk in tissues to be released under the influence of other hormones is one that may have to be considered here We also

Although we cannot say that we have completely elucidated any particular point as a result of this Colloquium, I am sure we have been able

So far, all these observations have not led us closer to understanding the basis of the biological activity of insulin, and here is obviously a most important field to be explored in the next few years. I think we can say that the biological activity of insulin in relation to its structure is a field that is hardly touched upon as yet.

Dr. Schulze's paper on the history of glucagon and Prof. Bürger's early work we greatly enjoyed, and to have Dr. Behren's important paper after that which dealt with glucagon as a subject for chemical investigation was very appropriate. Dr. Behrens emphasized that his paper was a progress report, and I am sure we are going to have a good deal of progress on this subject reported in the next year.

Prof. Sutherland introduced a subject that many of us as biochemists [illegible]

without coming to a definite conclusion. I thought Prof. de Duve made an excellent case for the existence of a significant action of insulin on the liver, which has been one of his loves for many years and which he has never deserted.

[illegible]

SUBJECT INDEX

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